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CCL18 and CXCL16: Traffic Control in Rheumatoid Arthritis

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CCL18 and CXCL16:
Traffic control in rheumatoid arthritis

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LIST OF ABBREVATIONS

AaMΦ	Alternatively activated macrophage
ACR	American college of rheumatology
ADAM	A disintegrin and metalloprotease
APC	Antigen presenting cell
ApoB	Apolipoprotein B
BSA	Bovine serum albumin
CaMΦ	Classically activated macrophage
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CK	Chemokine
Ct	Cycle threshold
CVD	Cardiovascular disease
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAS	Disease activity score
DC	Dendritic cell
dcSSc	Diffuse cutaneous systemic sclerosis
DM	Dermatomyositis
DMARD	Disease modifying anti-rheumatic drug
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme linked immunosorbent assay
EULAR	European league against rheumatism
FACS	Fluorescence activated cell sorting
FcR	Fc receptor
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GMCSF	Granulocyte monocyte colony stimulating factor
iDC	Immature dendritic cell
IFN	Interferon
IL	Interleukin

lcSSc	Limited cutaneous systemic sclerosis
LDL	Low density lipoprotein
MΦ	Macrophage
mDC	Mature dendritic cell
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MoDC	Monocyte derived dendritic cell
NHS	Normal human serum
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
oxLDL	Oxidized low density lipoprotein
p	Probability
PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocytes
PCR	Polymerase chain reaction
PI	Propidium iodide
PM	Polymyositis
PBGD	Porphobilinogeen deaminase
RA	Rheumatoid arthritis
RIA	Radio immuno assay
RF	Rheumatoid factor
RT	Reverse transcriptase
SD	Standard deviation
SEM	Standard error of the mean
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
SJC	Swollen joint count
SLE	Systemic lupus erythematosus
Sqrt	Square Root
SSc	Systemic sclerosis
ST	Synovial tissue
TCR	T cell receptor
TJC	Tender joint count
TLR	Toll-like receptor
TNF	Tumor necrosis factor

chapter 1

GENERAL INTRODUCTION

Auto-immunity

Inflammation is a key feature of a large variety of infectious and non-infectious diseases. Normally, the immune system acts as our defence mechanism, by means of directing an inflammatory response against foreign invaders, such as micro-organisms. Therefore, the ability to distinguish self from non-self and to interpret whether a given situation inflicts danger or not are key features of our immune system. When this mechanism fails, an inflammatory response to self antigens can occur, which is termed an auto-immune response. These auto-immune responses can be local and mild, but can also result in severe systemic auto-immune diseases. Auto-immune diseases comprise a heterogeneous group of mostly chronic diseases, which are all characterized by an ongoing inflammatory response that is directed towards the body's own constituents. The aetiology of auto-immune diseases is largely unknown. Formation of so-called auto-antigens, for instance through mutations, might play a role in igniting auto-immune responses or even drive the whole chronic auto-immune process. However, the exact antigen(s) to which the immune response is directed is/are still unknown in most auto-immune diseases. Another hypothesis is that auto-immune responses occur as a result of molecular structures of micro-organisms that resemble self molecules ("molecular mimicry"). Failure to down regulate immune responses during and after inflammation or inflammatory cascades running wild are alternative explanations that may, at least in part, contribute to auto-immunity. Although each auto-immune disease is directed against specific tissues and organs, signs and symptoms vary enormously among patients suffering from the same auto-immune disease and overlap between different auto-immune diseases is common, making a diagnosis often difficult. As the pathogenesis of most auto-immune diseases is largely unknown, their diagnosis is mostly based on a consensus of classification criteria, including a combination of clinical signs and symptoms and laboratory or radiological tests.

Rheumathoid Arthritis

Rheumatoid arthritis (RA) is, along with Systemic Lupus Erythematosus (SLE) and

ACR criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides fo the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Table 1 1987 ACR diagnostic criteria for rheumatoid arthritis

For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis is not to be made.

Multiple Sclerosis (MS), one of best known examples of an auto-immune disease. RA has a prevalence of approximately 1% worldwide and is therefore considered to be one of the most common autoimmune conditions. The hallmark of RA is chronic in flammation of the joints. The normal, healthy joint is formed by two bone structures, which are covered by a layer of cartilage to prevent direct bone to bone contact (figure 1a). The space between the bony structures, which is called the synovial cavity, is surrounded by the joint capsule. Within the synovial cavity, a layer of synovial tissue (ST) forms the inner lining of the joint cavity. This ST is responsible for the produc-

tion of synovial fluid (SF), which is critical for a smooth movement of the joint. In addition, the ST contains blood vessels and a variety of leukocytes. These leukocytes are critically involved in the eradication of apoptotic cells and cell debris, which originate from joint damages, e.g. due to traumatic events. The upper layer of this tissue, often referred to as the synovial lining, is a one cell layer thick linage consisting of macrophage like cells and fibroblast like cells. These two cell types are therefore often referred to as macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS). In inflamed joints of RA patients the synovial lining is hypertrophic with a massive influx of different leukocytes towards the sub-lining (figure 1b). Next to MLS and FLS, the ST consists of B and T lymphocytes and dendritic cells (DC) [1-4]. Interestingly, the inflamed ST in RA uniquely contains organized structures consisting of groups of B and T cells that mimic those found in activated secondary lymphoid organs, [5, 6]. Although their exact role is unknown, they might be involved in the chronic synovial inflammation which is characteristic for RA. In RA, this inflammation can eventually lead to cartilage damage and bone erosions (figure 1b), potentially resulting in severe disabilities for the patient. Next to pain relief, preventing bone erosions therefore is a major therapeutic goal in RA.

The clinical diagnosis RA is set by a number of disease criteria which were developed by the American College of Rheumatology (ACR) [7, 8] (table 1). The current treatment of RA rests on three pillars. First, non-steroidal anti-inflammatory drugs (NSAIDs) are used for pain relief. Second, disease modifying anti-rheumatoid drugs (DMARDs) are used to suppress disease activity and to prevent joint damage. Finally, biologicals comprise a novel group of disease modifying anti-rheumatic drugs which are now widely used in daily clinical practice. These agents specifically target a certain inflammatory cell-surface molecule or soluble mediators such as cytokines. The best known example is targeting tumour necrosis factor alpha (TNF-α) with monoclonal antibodies (infliximab, adalimumab) [9-11] or soluble receptor fusion proteins (etanercept) [12]. These agents, in combination with DMARDs, are now highly successful in the treatment of RA, resulting in a therapeutic response in about 70% of the patients [13]. The effect of treatment can be monitored with disease activity scores, such as the DAS-28 score [14]. This disease activity score evaluates 28 joints that are commonly involved in RA. The DAS-28 includes a visual analogue scale (VAS), the erythrocyte sedimentation rate (ESR) and the swollen and tender joint counts (SJC, TJC). As the DAS-28 is carefully validated in clinical practice, it allows tight monitoring of disease activity, which is a necessity when investigating potential novel therapeutic agents. Despite decades of intensive research, the exact pathogenesis of RA is still unknown. Although it is considered an auto-immune disease, no definitive human auto-antibody has been identified. Although rheumatoid factor (RF) was already discovered over 50 years ago [15-17] and is still used for diagnostic purposes, its

role in RA pathogenesis remains unknown, if at all present. More recently, antibodies against citrullinated peptides (anti-CCP's) were identified as highly specific for RA and therefore rapidly introduced as a novel diagnostic tool [18-20]. As for RF, the role of these antibodies in the pathogenesis however is still unclear. Currently, various players in the pathogenesis of RA are being investigated for their therapeutic potential. Especially soluble mediators such as cytokines are being widely explored as potential therapeutic agents, mostly in terms of neutralizing their biologic activity. Some of these cytokines are already used in the clinic or are currently under investigation in clinical trials. Examples include IL-1β, IL-6, IL-15 and IL-17 [21-33]. Another approach that is being explored is blocking cell-surface molecules. Neutralizing B cell activity with anti-CD20 antibodies (Rituximab) [34-36] and blocking the T cell co-stimulation receptor CD28 with CTLA-4Ig (Abatacept) [37-40] are two examples of strategies that have already reached the clinic. However, the efficacy of all these strategies varies tremendously, both among patients and during the disease course in individual patients. Therefore, there is still a great need for novel therapeutic strategies against RA. Moreover, both traditional DMARDs and biologicals can have (severe) side effects, which underscore the need for effective, but also safer therapeutic intervention strategies. More insight in the pathogenesis of RA is therefore critical for the identification of such novel therapeutic targets.

The APC-T cell interaction auto-immunity

As discussed above, failure of our immune system to distinguish self from non-self is the hallmark of auto-immunity. As different auto-immune diseases target different tissues and/or organs, the pathogenesis of a given auto-immune disease is characterized by its own key players. Nonetheless, leukocytes that migrate towards the site of inflammation are generally accepted to play a role in all inflammatory conditions, including auto-immune diseases. Two critical types of leucocytes involved in normal inflammation and auto-immunity are T lymphocytes and antigen presenting cells (APC), such as DC and macrophages. As DC are perfectly equipped for antigen presentation, they are often regarded as "professional APC" [41-44]. In their immature state, DC are specialized in taking up antigens. For this purpose, immature DC are active in macropinocytosis and express several different scavenger receptors on their surface, such as Fc gamma receptors (FcγR) and C-type-lectin receptors. In addition, DC express multiple receptors to interpret the local environment, including pattern recognition receptors, such as Toll-like receptors (TLR) and Nod-like receptors, to detect so called "danger signals". Upon receiving a stimulus ("danger signal"), immature DC lose their antigen uptake capacities and develop into mature DC. Classic examples of these maturation inducers are the TLR4 ligand lipopolysacharide (LPS) [45], CD40 ligation [46] or stimulation with inflammatory cytokine cocktails [47]. In turn,

these mature DC are ideally equipped for antigen presentation to T cells. DC are normally present in small amounts in the peripheral blood, which hampers large experiments with these cells. However, DC can be generated *in vitro* from blood monocytes by means of culturing these monocytes in the presence of IL-4 and GM-CSF [48]. The resulting monocyte derived DC (MoDC) functionally resemble blood DC in terms of antigen uptake and presentation capacities. A major task of APC is to present antigens taken up in the periphery in the context of major histocompatibility complexes (MHC) to T cells in the secondary lymphoid organs. For successful activation of naïve T cells at least three key signals are required (figure 2). Recognition of the MHC-peptide complex on the APC surface by the T cell receptor (TCR) on the T cell is referred to as signal 1. Signal 2 consists of co-stimulatory signals that are provided by the APC, like the co-stimulatory molecules B7.1 and B7.2 (CD80 and CD86) that interact with CD28 on the T cell surface. Finally, a third cytokine signal is needed to direct T cells into a specific differentiation pathway. Next to DC, macrophages and B cells also have antigen presenting capacities, although they are not as potent as DC. However, especially macrophages do have critical antigen uptake capacities and are therefore essential in the eradication of damaged leukocytes or other tissue components. Given the much higher numbers of these cells compared to DC, their cytokine secretion is responsible for a large proportion of local cytokine levels, which may subsequently influence the nature of immune responses. The exact pattern of cytokine secretion by macrophages is largely depending on how they are stimulated. For instance, stimulation with the Th1 cytokine IFN-γ ("classical activation") results in a distinct type of macrophage, compared to macrophages which are stimulate with the Th2 cytokines IL-4 or IL-13 ("alternative activation") [49]. T cells are the APC's receiving partner in antigen presentation and comprise a heterogeneous group of lymphocytes that play a central role in adaptive immune responses. Upon maturation, APC home to lymph nodes or other lymphoid structures to interact with T lymphocytes. Upon the encounter of new antigens, these proteins are presented as peptides to naïve T cells (Th0), which are T cells that have not yet encountered APC or differentiation signals. As a result of this interaction, Th0 cells are being directed into distinct types of T cells. T helper (Th) cells comprise one of these subsets, which can be further divided into Th1 and Th2 cells, depending on their cytokine secretion pattern. More recently, Th17 cells were described as a novel Th subset [50, 51]. In turn, Th cells activate B cells, which secrete immunoglobulins (Ig) upon differentiation into plasma cells, resulting in a fully developed adaptive immune response. Next to activated Th cells, Th0 cells can also differentiate into regulatory T cells (Tregs), which play a critical role in the inhibition of immune responses. In health, there is a tight balance between activated T cells and Tregs, designed to control immune responses. The potential effect of a disturbance of this balance may be auto-immunity, which is illustrated by recent findings in patients with the rare syndrome of immune dysregu-

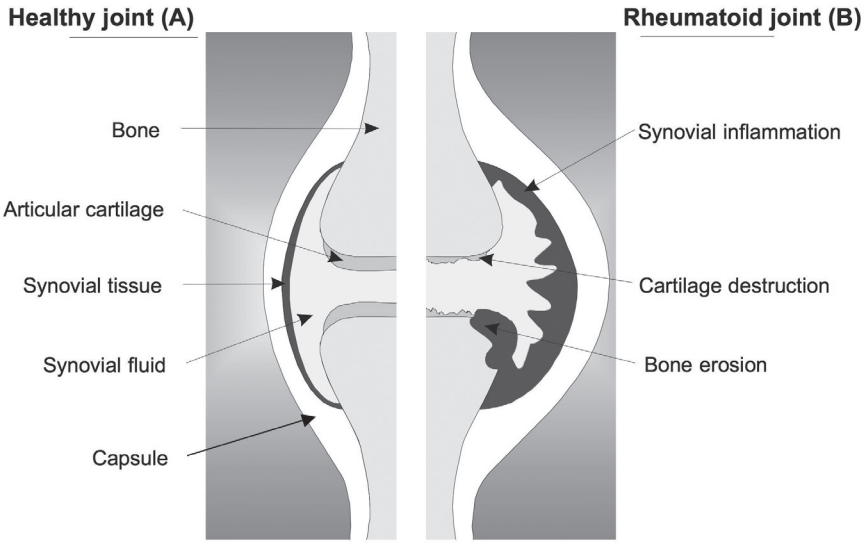

Figure 1
The joint in health and RA. Adapted from M Koenders:
"IL-17 and its relation to IL-1 and TNF in experimental arthritis"

lation, polyendocrinopathy, enteropathy, X-Linked (IPEX), who have been shown to have a mutation of the Treg marker FOXP3 [52]. Thus both APC and T cells play a key role in initiating and controlling adaptive immune responses. When a failure occurs anywhere in the cascade of antigen processing to T cell differentiation, this might lead to unwanted immune responses. It is therefore not surprising that the role of APC and T cells is being thoroughly investigated in various auto-immune diseases. Currently, a substantial amount of evidence indicates that both T cells and APC are key players in a variety of auto-immune diseases.

Chemokines orchestrate leukocyte migration

Interaction and communication between distinct cells of the immune system is a key feature of any immune response. As discussed above, APC and T cells are two cell types that drive immune responses upon their interaction, e.g. their cell-cell contact. In order to facilitate such an interaction, leukocytes secrete small chemotactic proteins which are termed chemokines. Chemokines comprise a large and ever growing family of molecules that facilitate leukocyte migration [53, 54]. To fulfil this task, they bind to and signal through 7-transmembrane spanning chemokine receptors on the surface of their target cells. Chemokines are classified according to their cysteine structure

and divided into four groups accordingly: CXC chemokines, CC chemokines, CXXC chemokines and C chemokines. The official nomenclature for chemokines is also derived from this classification, which made the chemokine system more transparent [55]. Before, several chemokines had up to 5 different names, according to their discovery by independent research groups, making the chemokine system unnecessarily complex. Next to the cysteine classification, another commonly used classification separates chemokines into inflammatory and homeostatic chemokines. However, these two functions cannot always be separated easily, making this classification less useful. The chemokine family is notorious for its redundancy and promiscuity, as several chemokines act as ligands for the same receptor. The chemokines CCL19 and CCL21 for instance both signal through chemokine receptor CCR7. In turn, this receptor is expressed on both mature DC and Th0 cells, which makes CCL19 or CCL21 a ligand for multiple distinct cell types. Next to chemotactic capacities, numerous other functions have been addressed to several chemokines. Examples of this diversity in functions will be discussed below for two specific chemokines, which form the key subject of investigation in this thesis. As described above, the interaction between APC and T cells largely determines the fate of the immune response. In order to facilitate this interaction, APC produce a specific set of chemokines which preferentially

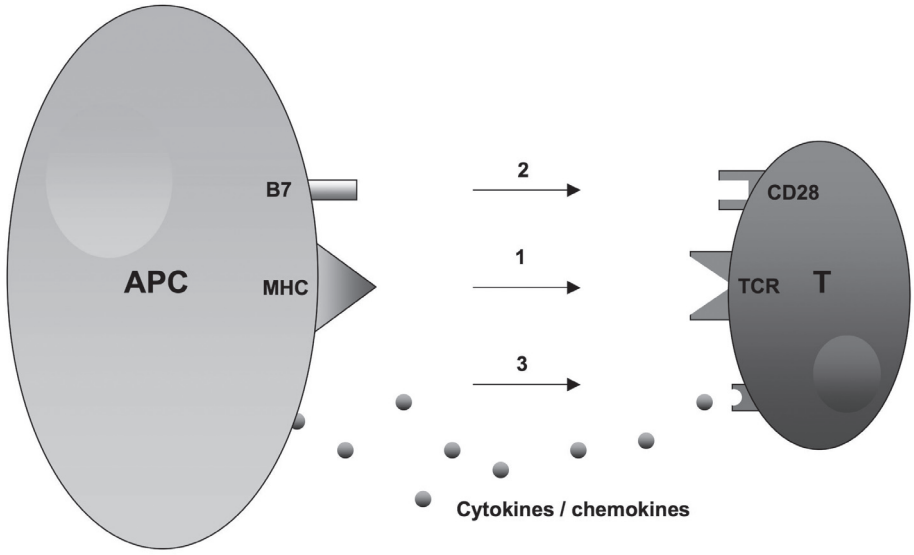

Figure 2
APC-T cell interaction

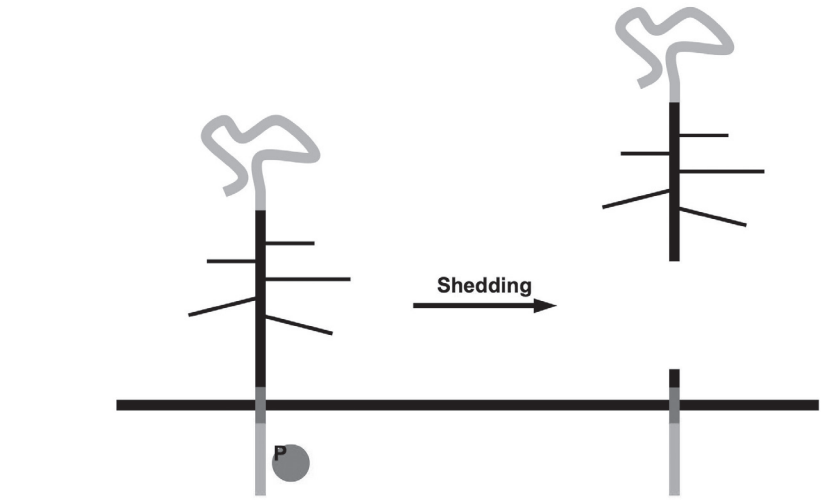

Figure 3
CXCL16 structure and shedding (Adapted from R. vd Voort, "oral presentation 2005")

attract T lymphocytes. Within this group of T cell attracting chemokines, each chemokine attracts its own group of T cell subsets. However, this is not a matter of black or white, as there also exists also redundancy among the T cell attracting chemokines. Moreover, novel subsets of T cells are still being identified, which by definition makes the picture incomplete. To date, a potential role for several T cell attracting chemokines has been suggested in the pathogenesis of chronic inflammatory and/or auto immune diseases. Examples include a potential role for CCL19, CCL21 in RA [6, 56, 57], CCL17 in SLE [58, 59] and CXCL10 in MS [60-62].

CC Chemokine Ligand 18

CC chemokine ligand 18 (CCL18) is a chemokine that was discovered in the late 90's by a number of independent groups, resulting in a variety of different names for this chemokine. In 1997, Adema and co-workers described a chemokine ligand which preferentially attracted CD45RA+ Th0 cells [63]. As this chemokine was predominantly expressed on DC, it was named "dendritic cell derived CC-chemokine 1" (DC-CK1). Interestingly, CCL18 was found to be expressed in germinal centres and T cell areas of secondary lymphoid organs, suggesting an important role in the

interaction between DC and Th0 cells. Almost simultaneously, Hieshima and colleagues described a chemokine that was expressed at high levels in the lung, which they subsequently named "pulmonary and activation regulated chemokine" (PARC) [64]. They found CCL18 mRNA to be expressed on alveolar macrophages, follicular DC and peripheral blood monocytes upon stimulation with LPS. In 1998, Kodelja and co-workers identified "alternative macrophage activation-associated CC-chemokine-1" (AMAC-1) [65], a chemokine that was expressed on mRNA level by alternatively activated macrophages [49, 66] *in vitro* and by alveolar macrophages *in vivo*. As MoDC expressed CCL18, it was intriguing that Langerhans cells, another well known DC-like cell, did not express CCL18 [67]. In addition, Wells and Peitsch and Guan *et al* reported a sequence that encoded for a novel CCL3-like chemokine with close homology to MIP-1α (SCYA3), which was named macrophage inflammatory protein 4 (MIP-4) (SCYA18) [68, 69]. According the official chemokine nomenclature, DC-CK1/PARC/AMAC-1/MIP-4 was renamed CCL18.

The CCL18 gene is located on chromosome 17q11.2, close to the location of most genes of the MIP family. The gene has a length of 7.2 kb, which is significantly longer than most other chemokine genes and encodes for a protein consisting of 69 amino-acids with a weight of 7.8 kD. This CCL18 protein has a 64% homology with CCL3. To date, CCL18 is still regarded as an orphan chemokine, since none of the currently identified chemokine receptors acts as a high affinity receptor for CCL18. However, it has been suggested that CCL18 may bind with low affinity to some of the known chemokine receptors, although the functional importance of these interactions need to be established. Another feature of CCL18 that hampers research on its role *in vivo* is the lack of a murine counterpart. Intriguingly however, it was recently claimed that recombinant human (Rh) and adenoviral (Ad) CCL18 were able to induce chemotaxis of murine lymphocytes [70].

The main function of CCL18 is (chemo) attraction of lymphocytes. CCL18 preferentially attracts CD45RA+, Th0 cells [63], but has also been reported to induce migration of activated CD3+ lymphocytes under specific conditions [64]. CCL18 mRNA is expressed *in vitro* by MoDC and alternatively activated macrophages while their precursors, peripheral blood monocytes, have been shown to express little or no CCL18 mRNA [63, 71]. *In vivo*, CCL18 is expressed on mRNA level by alveolar macrophages [64, 65] and germinal centre DC [63]. Next to DC and macrophages, CCL18 was also expressed by mantle zone B cells [72]. The exact regulation of CCL18 on myeloid cells is highly complex and still largely unknown, although CCL18 inducing effects of Th2 cytokines have been demonstrated on mRNA level [65]. In previous studies, we found that DC maturation using the TLR4 ligand LPS strongly increased CCL18 mRNA expression [57], which has been confirmed [71] and debated [65]. However, little is known about CCL18

regulation on the protein level, which off course is essential for a better understanding of the role of this interesting chemokine in health and disease. As is the case for several chemokines, chemo-attraction is not the only function of CCL18. CCL18 was recently described to play a role in collagen synthesis by lung and dermal fibroblasts, independently of fibroblast proliferation [73], suggesting CCL18 may be involved in the process of fibrosis. Interestingly, (pulmonary) fibrosis is a feature that occurs in various auto-immune diseases, which makes CCL18 an even more interesting subject of investigation in auto-immunity.

To date, a role for CCL18 has been suggested in a large variety of diseases, such as different forms of cancer [74-76], Gaucher's disease [77], pulmonary diseases [78, 79] and inflammatory/auto-immune conditions [57, 80]. An intriguing feature of CCL18 is that its circulating levels are relatively high, as most human studies so far describe as much as dozens of nanograms of CCL18 in serum or plasma of healthy individuals and even up to 1000 ng/ml in Gaucher's disease [77]. This allows it to be detected easily, making it a potentially interesting molecule as a prognostic marker for inflammatory diseases. Indeed, CCL18 has recently been put forward as a marker for Gaucher's disease [77].

CXC Chemokine ligand 16

CXC chemokine Ligand 16 (CXCL16) is one of the more recently identified members of the large and ever growing chemokine family and forms a unique chemokine-receptor couple with CXC Chemokine Receptor 6 (CXCR6). Remarkably, CXCL16 and CXCR6 were not initially linked to each other as a chemokine ligand and receptor pair. In 1997, Liao, Alkhatib and colleagues identified a novel gene encoding a chemokine receptor-like protein (STRL33) that acted as a co-receptor for HIV and the Simian Immunodeficiency Virus (SIV) [81, 82] on activated lymphocytes. Also in 1997, Deng and colleagues cloned two novel receptors that were used by SIV and HIV [83], one of which turned out to be the same molecule as STRL33, which later was often referred to as BONZO. In the same year, Loetscher and colleagues identified TYMSTR, a chemokine receptor-like protein expressed in CD4(+) T lymphocytes and a potential function as a HIV co-receptor [84]. A ligand for TYMSTR was not found among the already identified chemokines. Subsequent studies confirmed STRL33/BONZO to be expressed on peripheral blood lymphocytes and to act as a viral co-receptor in humans [85]. The relevance of CXCR6 as a chemokine receptor was discovered in 2000/2001, when Matloubian and Wilbanks identified a novel chemokine ligand named CXCL16 [86, 87]. Interestingly, the HIV co-receptor STRL33/BONZO/TYMSTR was identified as the receptor for CXCL16 and re-named CXCR6. CXCL16 was characterized as a trans-membrane chemokine consisting of different regions: an intracellular tail, a transmembrane part and an extracellular domain, consisting

of a mucin-like stalk and a chemokine domain (figure 3). In the same year, Shimaoka and co-workers reported the isolation of a cDNA clone from a library of stimulated THP-1 cells that encoded a 254 –amino acid protein of 30kD [88]. The corresponding gene was shown to be located on chromosome 17p13. This molecule was able to bind and internalize oxidized LDL (oxLDL) and was therefore named Scavenger Receptor that binds Phosphatidyl-Serine and Oxidized Lipoprotein (SR-PSOX) [88]. OxLDL induces the transformation of macrophages into foam cells [89] and the proposed role of SR-PSOX as a scavenger receptor for oxLDL was in line with its expression in atherosclerotic lesions and atheroma [90, 91]. Intriguingly, SR-PSOX proved to be identical to CXCL16, suggesting yet another chemokine with a dual role. Further insights in the regulation of CXCL16 revealed that membrane bound CXCL16 could be cleaved by A Disintegrin And Metalloprotase 10 (ADAM-10). This cleavage released the chemokine moiety of CXCL16 and allowed it to serve as a chemokine ligand [92, 93]. Next to chemo attraction and oxLDL binding, CXCL16 was reported to mediate bacterial phagocytosis through its chemokine domain [94]. Finally, CXCL16 was reported to not only attract leucocytes, but also to mediate adhesion of leucocytes [95].

To date, CXCL16 expression and secretion has been described on a variety of different cells, such as APC, smooth muscle cells and fibroblasts [88, 92, 96]. Regarding the regulation of CXCL16, the Th1 cytokine IFN-γ has been claimed to induce CXCL16 production by myeloid cells under specific circumstances [96, 97]. Next to myeloid DC, CXCL16 expression has been reported on plasmacytoid dendritic cells (pDC) [98], but this finding was not consistent in different studies [99]. CXCL16 mRNA expression has been reported once on lymphocytes [100], but this finding has not been confirmed and no functional attraction of CXCR6 positive cells has been shown so far. The CXCL16 receptor CXCR6 is expressed mainly on activated T cells [85, 87]. Given the expression profile of the CXCR6-CXCL16 couple, it is conceivable that CXCL16 and CXCR6 are involved in facilitating APC-T cell interactions. However, in contrast to CCL18, CXCL16 recruits already activated T cells instead of Th0 cells. Regarding CXCR6 regulation, the cytokine IL-15 has been demonstrated to induce CXCR6 expression on lymphocytes [101, 102]. Apart from these initial observations, still little is known regarding the regulation of the expression of CXCR6. Therefore, it will be interesting to study the regulation and expression of CXCR6 and its ligand CXCL16 in distinct pathological conditions, including RA.

CCL18 and CXCL16 in Rheumatoid Arthritis

As mentioned above, CCL18 has been suggested to play a role in the pathogenesis of several inflammatory diseases. As we are particularly interested in the APC-T cell interaction in RA, we evaluated the potential role of several T cell attracting chemo-

kines in RA. For this purpose, we started off with a large panel of chemokines secreted by APC, of which CCL18 came out as a T cell attracting chemokine of particular interest [57]. In line with this finding, the presence of CCL18 in the ST of RA patients was described by us and others [57, 74]. Interestingly, CCL18 co-localized with mature DC in secondary lymphoid follicles [57]. To date, a substantial amount of data points toward the enrichment of DC and macrophages in the ST, which both may account for abundant CCL18 secretion in RA joints. Given its Th0 cell attracting properties, its secretion by APC and its presence in RA joints, CCL18 is an interesting subject for further investigation in RA, both for its role in the pathogenesis and for its potential as a clinical marker, given its high circulating levels *in vivo*.

As CXCL16 is well appreciated as a chemotactic factor for activated T cells that bear CXCR6, its role in inflammatory disease has been both been investigated and suggested in a large variety of chronic inflammatory diseases, such as inflammatory diseases in the lung [102, 103]. The first indication that CXCR6 might be of importance in RA came from the observation that CXCR6 was abundantly expressed on both CD4+ and CD8+ T cells in rheumatoid ST [104]. Next to its chemotactic properties, the ability of membrane-bound CXCL16 to internalize oxLDL might be of interest in RA, as patients with RA have an increased morbidity and mortality due to cardio vascular disease, compared with the general population [105], that is accompanied by an increase in several cardiovascular risk factors [106-108].

Outline of this thesis

The chemokines CCL18 and CXCL16 are both T cell attracting chemokines which are expressed by APC. Moreover, there already is some initial evidence supporting a potential role for these chemokines in RA [57, 80, 104]. The general aim of this thesis was therefore to further explore the potential role of CCL18 and CXCL16 in RA. For this purpose, we investigated the regulation of these chemokines in healthy donors and RA patients, their potential role in RA and whether their circulating levels *in vivo* correlated with clinical disease parameters. We recently demonstrated that CCL18 mRNA production was significantly enhanced in mature DC derived from patients with RA [57]. In *chapter 2*, we further investigated the level of CCL18 expression in RA DC and studied how neutralization of TNF-α, which is currently applied as a treatment for RA, would affect CCL18 mRNA production by DC. In addition, we investigated whether TNF-α neutralization would affect cytokine secretion of DC maturation in general. As little is known about the regulation of CCL18 at the protein level, we developed an ELISA and investigated the regulation of CCL18 protein expression in myeloid cells in *chapter 3*. As CCL18 expression was shown to be enhanced in RA ST, we additionally investigated the effects of RA SF on CCL18 secre-

tion by these myeloid cells. In *chapter 4*, we explored the expression and function of the chemokine CXCL16 in RA joints. As for CCL18, little was known about the regulation of CXCL16 by myeloid cells. We therefore aimed to investigate the regulation of CXCL16 by monocytes, DC and macrophages in *chapter 5*. Both CCL18 and CXCL16 have relatively high circulating levels which makes them potentially interesting as clinical markers. Moreover, circulating levels in the disease course of RA may provide important clues for the understanding of the role of these chemokines in the pathogenesis of RA. For this reason, we set out to investigate circulating levels of CCL18 and CXCL16 in a prospective cohort of RA patients and a cohort of RA patients that were treated with anti-TNF-α in *chapter 6*. CXCL16 has been proposed to act as a chemokine and as a receptor for oxLDL. This might be of particular interest in case of inflammatory disease like RA, in which cardiovascular morbidity is enhanced [105, 106]. We therefore investigated the potential of soluble CXCL16 to bind oxLDL and its correlation with circulating markers of atherosclerosis in patients with RA in *chapter 7*. In the final chapter, *chapter 8*, the expression, secretion, regulation and circulating levels of the T cell attracting chemokines CCL18 and CXCL16 are discussed in the context of RA.

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chapter 2

INHIBITION OF TNF-ALPHA DURING MATURATION OF DENDRITIC CELLS RESULTS IN THE DEVELOPMENT OF SEMI-MATURE CELLS.

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Abstract

Background: Dendritic cells orchestrate pivotal immunological processes mediated by the production of cytokines and chemokines.

Objective: To assess whether neutralisation of tumour necrosis factor α (TNF-α) during maturation of dendritic cells affects their phenotype and behaviour, which might explain the beneficial effects of TNF-α neutralisation in rheumatoid arthritis.

Methods: Immature and fully matured dendritic cells were cultured from blood monocytes from patients with rheumatoid arthritis and healthy controls following standardised protocols. TNF-α was neutralised by addition of the p55 soluble TNF-α, PEGsTNF-RI. The effect of TNF-α neutralisation on the phenotype (CD14, CD16, CD32, CD64, CD80, CD83, CD86 and MHC) of dendritic cells was investigated by flow cytometry. Expression of chemokines (CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8) and production of IL-1β and IL-6 were examined.

Results: Neutralisation of TNF-α during the differentiation and/or maturation of DC did not result in an altered DC phenotype in the rheumatoid arthritis patients or the healthy controls. In contrast, the expression of CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8 by DC was significantly reduced when TNF-α activity was inhibited during lipopolysacharide triggered dendritic cell maturation. The production of IL-1β and IL-6 by matured dendritic cells was inhibited by PEGsTNF-RI.

Conclusion: Inhibition of TNF-α activity during DC maturation lead to the development of semi-mature cells. These data suggest a novel pathway by which the neutralisation of TNF-α might exert its therapeutic effects.

Introduction

Rheumatoid Arthritis (RA) is a chronic autoimmune disease characterised by a symmetrical polyarthritis of the synovial joints leading to destruction of the cartilage and underlying bone. Although the exact mechanisms are still unclear, there is a large body of evidence suggesting a critical role for inflammatory mediators such as cytokines and chemokines [1-3]. Pro- and anti-inflammatory cytokines, and more importantly the balance between these opposing groups, are likely to play a crucial role in the onset and perpetuation of RA [4-6]. In this disease, interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) are thought to be the key mediators as concentrations of these cytokines are significantly elevated in the peripheral blood and the synovial compartment of RA patients and blockade of these mediators has proven to be a highly successful form of treatment [7-9].

Chemokines are members of a superfamily of proteins that ensure that cell trafficking occurs in a proper temporal and spatial fashion [10]. There is evidence for an important role for the chemokines interleukin 8 (IL-8; CXCL8), macrophage inflammatory protein α (MIP-1α; CCL3) and fractalkine (CX3CL1) in RA [11-13]. The fact that the blocking of chemokine receptor 1 in RA patients results in a significant reduction in synovial cellularity further substantiates the important role of chemokines in synovial inflammation [14]. Previous work from our group demonstrated that DC from RA patients express significantly higher levels of the chemokines CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8 than those from healthy donors [15]. In particular, chemokines CCL17, CCL18 and CCL19 were expressed at high levels, which is intriguing as these chemokines largely orchestrate the attraction of T cell subsets and might thus play an important role in RA pathology.

In RA, both the synovial tissue and synovial fluid contain large numbers of inflammatory cells, including T cells, fibroblasts, monocytes, macrophages and dendritic cells (DC) [16-18]. DC are professional antigen presenting cells (APC) that are crucial in directing tolerance and immunity as well as the initiation of innate and adaptive immune responses [19, 20]. Immature DC reside in the periphery and are specialised in uptake and processing of antigens. Maturation of these cells is triggered by a multitude of pro-inflammatory stimuli and which coincides with major changes in DC phenotype and a loss of the ability to capture antigens [21]. To date, a large body of evidence points to an important role for DC in synovial inflammation [22-26].

For an optimal DC function the phenotypic characteristics (signals 1 and 2) are crucial and are obligatory for T cell activation [27-29], while the capacity of mature DC to prime naive T cells and promote their differentiation is attributed to their cytokine secretion pattern (signal 3) [30]. While immature DC induce tolerance in the steady

state, mature DC induce antigen-specific immunity [27, 31, 32]. A disturbed balance of pro-inflammatory cytokines, which is undoubtedly present in RA, might therefore contribute to an enhanced DC maturation, culminating in a disturbed balance between tolerance and (auto)immunity in RA.

The potential role of TNF-α in DC biology, along with the clinical effects of TNF-α blockade in RA, prompted us to investigate the effects of TNF-α blockade on DC development. We hypothesised that the inhibition of TNF-α during DC development would interfere with DC maturation and the production of inflammatory mediators, so leading to restoration of immunological balance. We show here that neutralisation of TNF-α during DC maturation leads to the development of semi-mature DC.

Methods

Patients

Eleven patients with active RA and 10 healthy volunteer controls were enrolled in the study. All patients fulfilled the American College of Rheumatology (ACR) criteria for RA and gave informed consent for the study [33]. We excluded patients who received therapy with systemic steroids or biological agents. All patients were treated with disease modifying anti rheumatic drugs alone or in combination with non-steroidal anti-inflammatory drugs. To assess disease activity, the DAS28 score was used [34]. The Medical Ethics Committee of the University Medical Centre Nijmegen approved the study protocol.

Dendritic cell cultures

For cell cultures, 50 ml blood was taken from the RA patients and the controls intro 10ml heparinised Vacutainer tubes. DC were cultured from peripheral blood monocytes using standardised protocols, essentially the same as previously described [35]. In brief, the procedures were as follows: Peripheral blood mononuclear cells were isolated by means of a Ficoll gradient centrifugation gradient (Ficoll Paque, Amersham Biosciences, Uppsala, Sweden). After several stringent wash steps, cells were incubated in six-well plates (Costar, Badhoevedorp, The Netherlands) for one hour at 37°C to allow adherence of monocytes. Thereafter, adherent cells were cultured in medium (RPMI-1640 (Dutch modification), Life Technologies, Grand Island, USA), supplemented with glutamine, antibiotics/antimycotics (both from Life Technologies, Grand Island, USA) and 10% fetal calf serum (FCS) for 6 days in presence of interleukin 4 (IL-4) (500U/ml) and granulocyte macrophage colony stimulating factor (GM-CSF) (800U/ml) (Schering-Plough, Amstelveen, The Netherlands). On day 6, a proportion of the iDC was harvested for analysis of the immature state. To obtain full maturation, the remaining cells were cultured for two more consecutive days in the

presence of 2µg/ml E.coli lipopolysacharide (LPS) (Sigma-Aldrich, St. Louis, USA). On day 8, fully mature DC were harvested and studied.

Effective blockade of TNF-α activity in DC cultures

For TNF-α blockade, we used a p55 soluble TNF-α receptor (PEGsTNF-RI), kindly gifted to us by AMGEN®. To assess the amount of PEGsTNF-RI needed for full inhibition of TNF-α activity during the culture of DC, we determined the maximum production of TNF-α by matured DC originating from previous experiments from our group [35]. To achieve efficient neutralisation of TNF-α we added an 1000-fold excess of PEGsTNF-RI to the cultures. On day 0, 3 and 6 PEGsTNF-RI was added to the culture medium to examine its effect on the differentiation and maturation. As a control, carrier fluid that was provided by AMGEN® was added to the cultures where appropriate.

To check whether a 1000-fold excess of PEGsTNF-RI was sufficient for maximum inhibition of TNF-α, we carried out a bio-assay using a 3T3 luciferase reporter cell line that is highly sensible for mediators that trigger NFκB signalling including TNF-α and IL-1β [36]. As an initial step, we carried out a dose-response curve to test whether the PEGsTNF-RI was capable of the neutralisation of TNF-α and to study the value of our bio-assay for this purpose. Second, the supernatant obtained from the DC cultured with or without PEGsTNF-RI was added to the 3T3 cells and the luciferase response was studied. Another 1000-fold excess of PEGsTNF-RI was added to the system to test whether maximum inhibition of TNF-α was achieved in our experimental setup.

Determination of DC phenotype using FACS techniques

The phenotype of DC was characterised using flowcytometry techniques (fluorescence activated cell sorting, FACS) (FACSCalibur®, Becton-Dickinson, San Jose, USA). For this aim, DC were harvested and collected by centrifugation and further processed on melting ice. Cells were diluted in buffer solution (phosphate buffered saline (PBS) with 1% bovine albumine, pH 7.4) in a concentration of $1x10^6$ cells per ml and plated in v-shaped 96-wells plates ($1x10^5$ cells per plate). Cells were labelled with monoclonal mouse-anti human antibodies against the monocyte marker CD14, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (all from Dako, Glostrup, Denmark), DC-SIGN [37], the co-stimulatory molecules CD80 (Becton Dickinson, Mountain View, USA) and CD86 (PharMingen, San Diego, USA), the mature DC marker CD83 (Beckman Coulter, Mijdrecht, The Netherlands), MHC-I (clone W6/32) and MHC-II (clone q1513), with mouse-isotype control and incubated at 4°C for 45 minutes. Cells were then washed and labelled with goat anti-mouse FITC (Zymed Laboratories, South San Francisco, USA) as a secondary antibody. After another 30 minutes' incubation at 4°C, cells were again washed, diluted in buffer solution and transferred into FACS tubes. Cell phenotype was then analysed using FACS .

Table 1. Primers and Taqman™ probes used for real-time quantitative PCR analysis [40]

	primer		probe	
Chemokine	5'end	3'end	5'end	3'end
DK-CK1 (CCL18)	CCTGGAGGCCACCTCTTCTAA TGCAGCTCAACAATAGAAATCAATT		AGTCCCATCTGCTATGCCCAGCCAC	
ELC (CCL19)	CAGAGGACCTCAGCCAAGATG TTCACAATGCTTGACTCGGACT		CCTATGACCGTGCAGAGGGAGCCC	
IL-8 (CXCL8)	AGAAGTTTTTGAAGAGGGCTGAGA CAGACCCACACAATACATGAAGTG		TCCAAGAATCAGTGAAGATGCCAGTGAAACTT	
MDC (CCL22)	GTCCTGTTCCCATCAGCGAT CAGGCTGGAGAGAGAGATGGA		CCATGACTCCCCACTGCCCTAAGCT	
MIP 1α (CCL3)	TGTGTTTGTGATTGTTTGCTCTGA TGGTGCCATGACTGCCTACA		CCTTCCCTCACACCGCGTCTGG	
TARC (CCL17)	GCAAAGCCTTGAGAGGTCTTGA CGGTGGAGGTCCCAGGTSGT		CCTCCTCACCCCAGACTCCTGACTGTC	
GAPDH	GAAGGTGAAGGTCGGAGT AGATGGTGATGGGATTTC		CAAGCTTCCCGTTCTCAGCC	
PBGD	GGCAATGCGGCTGCAA GGGTACCCACGCGAATCAC		CTCATCTTTGGGCTGTTTTCTTCCGCC	

Radio Immuno Assay (RIA) for IL-1β production levels

When the DC were harvested, the supernatants were collected and stored for the measurement of cytokines. Polyclonal antibodies for IL-1β were kindly provided by Sclavo (Siena, Italy). Human recombinant IL-1β was radio-labeled by using the chloramine-T method [38]. Briefly, all samples and standards were prepared and mixed with a standard buffer which contains 13mM NA2 EDTA, 0.02% sodium azide, 0,25% bovine serum albumin (Boehring, Marburg, Germany) and inactivating units aprotinine, pH 7.4 (Bayer, Leverkussen, Germany). For measurement of IL-1β in supernatants, 10µl of sample or standard was added to the buffer. The mixture was incubated for one day at room temperature. After the addition of tracer (≈ 7,000 dpm/100µl) the incubation was continued for two more days. Separation of bound and free tracer was achieved by the addition of 100 µl of a separation agent containing sheep anti-rabbit immunoglobulin G and 0.01% rabbit immunoglobulin G (Sigma). After incubation for 30 minutes, the antibody complex was completely precipitated by the addition of 1 ml 7.5% polyethylene glycol 6000 (Merck, Darmstadt, Germany). The range of the standard curve was 20 to 3000 pg/ml with a sensitivity of 40 pg/ml. To minimise inter-assay variations, all samples from the same experiments were analysed in the same run in duplicate. The inter-assay variation of our RIA is estimated at ≤ 15%, whereas the intra-assay variation is ≤ 10%.

Table 2. Phenotypic characteristics after LPS mediated maturation with and without PEGsTNF-RI

	Healthy controls				RA patients			
	LPS alone		LPS + PEGsTNF-RI		LPS alone		LPS + PEGsTNF-RI	
Marker	% positive cells	MFI	% positive cells	MFI	% positive cells	MFI	% positive cells	MFI
CD14	10 (4)	11 (3)	11 (5)	12 (4)	10 (3)	13 (4)	13 (4)	15 (5)
FcγRI	8 (2)	13 (4)	12 (2)	14 (2)	10 (3)	15 (4)	11 (4)	16 (4)
FcγRII	22 (4)	43 (8)	24 (7)	47 (8)	53 (6) *	83 (8)*	51 (9)*	84 (7)*
FcγRIII	12 (4)	17 (7)	14 (5)	16 (5)	26 (6) *	19 (4)	27 (5)*	18 (7)
DC-SIGN	71 (10)	29 (5)	71 (11)	27 (3)	76 (10)	26 (3)	79 (9)	29 (7)
CD80	97 (1)	62 (8)	94 (2)	53 (10)	95 (3)	81 (15)	95 (2)	63 (14)
CD83	87 (4)	30 (4)	77 (5)	25 (4)	87 (4)	38 (7)	77 (7)	32 (5)
CD86	97 (1)	113 (19)	94 (3)	99 (15)	97 (1)	108 (16)	89 (5)	102 (16)
MHC-I	96 (2)	112 (29)	95 (2)	92 (21)	96 (2)	106 (26)	94 (3)	149 (61)
MHC-II	98 (1)	312(116)	99 (1)	331 (98)	98 (1)	343 (112)	97 (2)	388 (123)

Values are mean (SD)
MHC-II = HLA-DR/DP
* Significant difference between DC from healthy controls and from RA patients $p<0.05$.

Enzyme Linked Immuno Sorbent Assay (ELISA) for IL-6 production levels
IL-6 was measured by using a commercially available enzyme linked immunosorbent assay (ELISA) (Pelikine CompactTM human IL-6 ELISA kit, CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions [39].

Primers and probes for chemokine production measurements
The sequence of the primers (Life technologies) and TaqmanTM probes (PE Biosystems, Branchburg, New Jersey) used in this study are given in table 1. The chemokine-specific probes were labelled at the 5' end with a FAM fluorescent group and at the 3' end with a TAMRA quencher group. The probes specific for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogeen deaminase (PBGD) were labelled with a VIC fluorescent group at the 5' end.

Real Time PCR for chemokine production levels
Expression levels of CCL17, CCL18, CCL19, CCL3, CCL22 and CXCL8 was measured in six patients and six controls using real-time polymerase chain reaction (PCR) techniques. After isolation of the DC, the cells were centrifuged and the pellet was solved in the Trizol reagent and stored at -70°C. Thereafter, RNA was extracted using reverse transcriptase polymerase chain reaction (RT-PCR) with 1 microgram of RNA.

OligoT primers were used for reversed transcription of mRNA. For PCR amplification, 5% of the cDNA was used. The standard PCR buffer that was used, contained 200μM dNTP's, 0,1μM forward and reverse primer and 1 unit Taq polymerase. We followed TaqmanTM assay instructions for PCR procedures, with an end concentration of 175nM probe and 600nM primers. All PCR amplifications were done on a ABI/PRISM 7700 sequence detector system. This system produces a real-time amplification plot based upon the normalised fluorescence signal. The expression levels of the chemokines were related to the expression level of PBGD, a housekeeping gene with intermediate expression levels. Another housekeeping gene GAPDH was used as an internal control for the amount of cDNA in each individual.

Statistical analysis

Comparisons between groups were assessed using paired t tests or Wilcoxon's signed rank test as appropriate. For statistical analysis, the GraphPad Prism® version 4 statistical program was used. Probability (p) values < 0.05 were considered significant.

Results

TNF-α activity inhibition

The maximum production of TNF-α by mature DC was around 5000 pg/ml, in line with a previous study [35]. To determine the concentration of PEGsTNF-RI needed for an optimal TNF-α activity inhibition, we added different concentrations of the soluble TNF-α receptor to 3T3 reporter cells which were stimulated with 1ng/ml TNF-α (figure 1a). A PEGsTNF-RI concentration of 100 ng/ml decreased the luciferase response with 56% ($p < 0.001$), while maximum (86%) inhibition of TNF-α activity was achieved by 1000 ng/ml PEGsTNF-R (a 1000-fold excess). A further increase of PEGsTNF-RI did not result in a greater inhibition of the luciferase response. In our supernatants obtained from fully matured DC, there was a 33% inhibition of the luciferase response after the addition of a 1000-fold excess (10^4 ng/ml) of PEGsTNF-RI (figure 1b). A further increase of 1000-fold (10^4 μg/ml) in PEGsTNF-RI did not result in a further decrease in the luciferase response, indicating that a maximum neutralisation of TNF-α in the supernatant had been achieved. The remaining luciferase response is likely to be caused by other NFκB activating cytokines in the supernatant.

Effect of TNF-α blockade on dendritic cell differentiation and maturation

We next investigated the effect of TNF-α neutralisation during monocyte differentiation into immature DC. We added PEGsTNF-RI (1000-fold excess) to the monocytes on day 0 and day 3 during DC development and compared the phenotype of the resulting immature DC with cells cultured under the same conditions but in the absence of PEGsTNF-RI. Both cell cultures expressed high levels of FcγRI, II and

III, intermediate levels of the markers CD80, CD86 and MHC-I&II and low levels of CD14 and CD83, typical phenotypical features of the immature DC [40, 41]. In line with previous observations, the expression of FcγRII was higher on immature DC from RA patients (henceforth referred to as RADC) when compared with that of healthy controls (CDC) (table 2). However, no differences in DC phenotype were detected between cells cultured with and without PEGsTNF-RI, either in the rheumatoid group or in the control group, nor did the addition of PEGsTNF-RI affect the phenotype after LPS mediated maturation in either group. In contrast, a clearly increased expression of co-stimulatory molecules and MHC was observed in both groups, whereas expression of FcγRs was down-regulated, resulting in a mature DC phenotype (table 2) even when TNF-α was inhibited to the maximum extend.

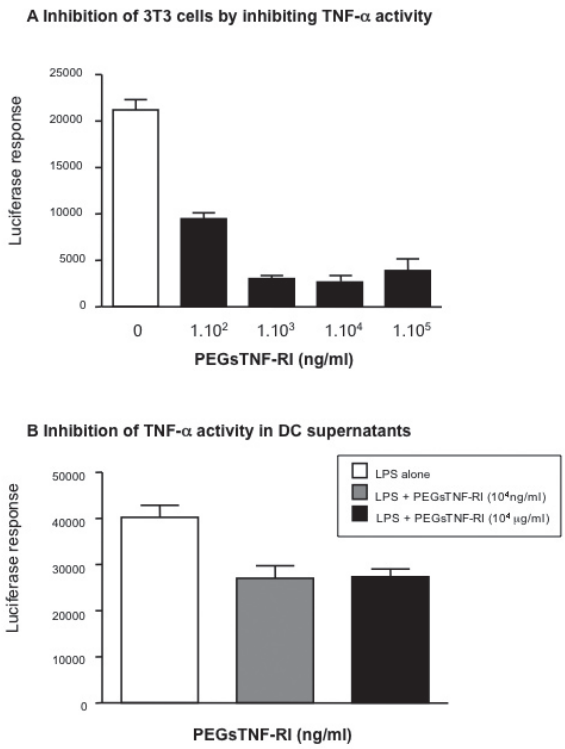


Figure 1.

Inhibition of tumour necrosis factor (TNF) activity by the addition of the p55 soluble TNF receptor PEGsTNFRI.
(A) Inhibition of 3T3 cells (luciferase response) stimulated by TNF-alpha (1 ng/ml) following the addition of various concentrations of PEGsTNFRI. (B) Neutralisation of TNF-alpha activity in the supernatant of mature dendritic cells by addition of PEGsTNFRI. A 1000-fold excess of PEGsTNFRI was sufficient to provide maximum inhibition of TNF-alpha activity, as a further 1000-fold excess did not cause any additional decrease in luciferase. (DC, dendritic cell).

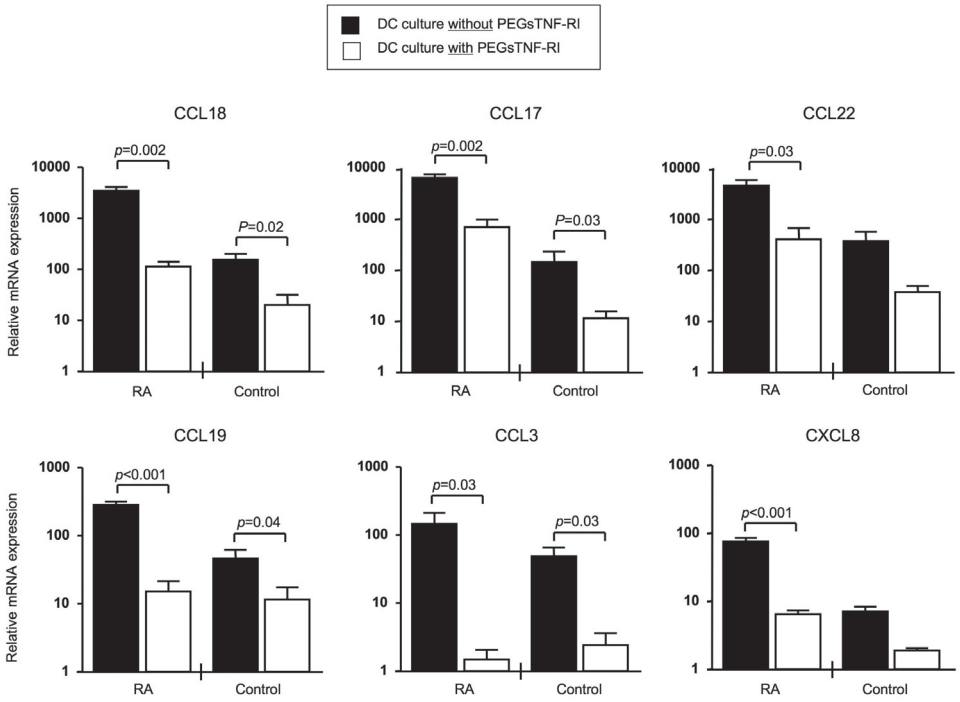


Figure 2.

Decrease in chemokine expression by mature dendritic cells when TNF-alpha activity is inhibited.

Expression of the chemokines CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8 by lipopolysacharide matured DC was measured using Real-Time PCR techniques. A clear decrease in chemokine expression was observed when TNF-alpha activity was inhibited during the maturation process in both RA patients and healthy controls.

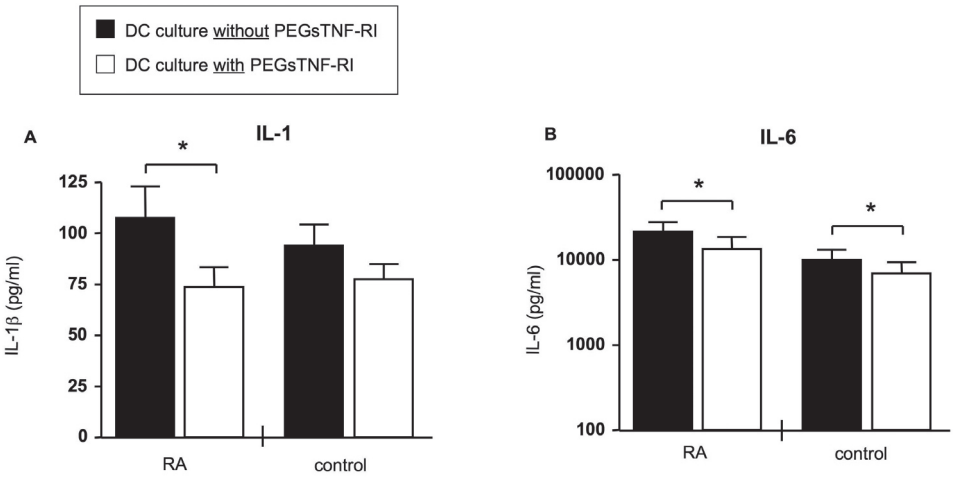
Decreased expression of CCL17, CCL18, CCL19, CCL3, CCL22 and CXCL8 after TNF-α neutralisation.

As we found previously that RADC expressed significantly higher levels of chemokines and pro-inflammatory cytokines than DC from healthy controls, we examined whether this process is TNF-α driven. To do this, we investigated the effect of TNF-α neutralisation on the expression of several DC-specific and non-specific chemokines during LPS triggered DC maturation. Fully matured RADC expressed significantly higher levels of the chemokines CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8 than CDC (Figure 2). Intriguingly, the neutralisation of TNF-α during maturation of RADC resulted in a significantly decreased production of CCL18 (mean (SEM), pg/ml: 3514 (581) v 114 (27), p=0.002), CCL17 (7261 (935) v 424 (178), p=0.002), CCL22 (4636 (1278) v 405 (268), p=0.03), CCL19 (281 (35) v 15 (6), p<0.001), CCL3 147 (66) v 1.5 (1.0), p=0.03) and CXCL8 (76 (10) v 7 (1), p<0.001). A similar trend

Figure 3.

Inhibition of TNF-alpha during DC maturation resulted in a decreased production of IL-1 and IL-6.

(A) Production of IL-1 , (B) IL-6 secretion. Both pro-inflammatory mediators were significantly reduced when TNF-alpha was neutralised during DC maturation triggered by the addition of LPS. Although the production of both IL-1 and IL-6 was significantly greater by DC from RA patients, the decrease in production by blocking TNF-alpha was similar in both groups.



was observed on chemokine expression by DC from healthy donors although this effect failed to reach statistical significance for CCL22 (p=0.09) and CXCL8 (p=0.06). Whereas inhibition of TNF-α activity in DC cultures from rheumatoid patients led to a significant decrease in chemokine expression, expression of CCL17, CCL18, CCL19, CXCL8 and CCL22 remained increased compared with healthy controls. This suggests that chemokine expression is not driven by TNF-α alone.

Decreased production of IL-1β and IL-6 by mature dendritic cells after TNF-α inhibition. As IL-1 and IL-6 are defined as key inflammatory mediators in RA, we investigated whether inhibition of TNF-α during DC culture influenced the secretion of these mediators. As with the expression of chemokines, the secretion of both IL-1 (mean (SEM):108 (16) v 74 (10) pg/ml, p=0.02) and IL-6 (21282 (6543) v 13482 (5037) pg/ml, p=0.007) decreased significantly when TNF-α was neutralised during maturation of RADC (figure 3). Similarly, the addition of PEGsTNF-RI during maturation of CDC also resulted in a decreased production of IL-6 (10040 (3200) v 6970 (2100), p=0.005), although the decrease in IL-1 secretion was not significant (94 (10) v 78 (8) pg/ml , p=0.08).

Discussion

Our results show that the neutralisation of TNF-α during DC maturation does not inhibit DC maturation in terms of phenotype but does lead to a decreased production of inflammatory mediators that reflects the development of the so called "semi-mature" DC. The presence of these semi-mature DC might at least partially explain the therapeutic effects of neutralisating TNF-α *in vivo*.

Autoimmunity is characterised by a loss of tolerance to the body's own constituents that results in a destructive process directed to a specific organ. The major goal in the treatment of autoimmune diseases would be the inhibition of APC function and the generation of tolerogenic DC [32]. As RA is an autoimmune disease, DC are an attractive therapeutic target. Their importance in synovial inflammation was suggested by the fact that mature DC are present in synovial tissue of RA patients and are located strategically in well organised structures [22, 23, 26]. Furthermore, DC are able to trigger and abrogate experimental arthritis, depending on the time of administration and the experimental set-up [24, 25]. Modulation of DC in RA might therefore be used to combat autoimmunity as has already been achieved successfully in other autoimmune diseases [42].

DC are professional antigen presenting cells that play a critical role in the fine tuning of the balance between immunity versus tolerance. Whilst immature DC are perfectly adepted for antigen uptake and processing, their maturation leads to functional changes that enhance their ability of DC to attract and activate T cells [31, 43]. However, recent evidence has challenged this oversimplified model of immature and mature DC [44-46]. Lutz and co-workers suggested that cytokines produced by fully matured DC are crucial for T cell immunity and proposed the theory of so called semi-mature DC, which resemble mature DC in terms of their phenotype but produce low levels inflammatory mediators and are critical for induction of tolerance [45]. However, the level of T cell stimulation remains strongly dependent on the signals mediated through various molecules expressed on the DC cell surface. The first signal accounts for the specificity of the immune response and involves the engagement of T cell receptors by an appropriate peptide-MHC complex. Second, interaction between co-stimulatory molecules (signal 2) is required and determine the quality and fate of the immune response. Finally, pro-inflammatory mediators are thought to function as additional signals (third signal) which, direct T cells in harmony with signals received through the TCR and co-stimulatory molecules [30]. The type and quantity of these given signals is likely to determine the fate of the T cells. It is therefore tempting to speculate that these so called semi-mature DC drive the immune response toward the induction of T cell anergy.

We have recently shown that DC obtained from RA patients produce higher levels of pro-inflammatory mediators than those from healthy controls [35]. This suggests

that in RA, DC possess a lower threshold for activation which, might lead to an altered balance between tolerance and (auto)immunity. The fact that the production of inflammatory mediators by DC is at least partly inhibited by the neutralisation of TNF-α during DC maturation suggests that this might be one of explanation for the clinical success of TNF-α blockade. However, the production of inflammatory mediators by DC in RA remains higher despite the inhibition of TNF-α, which suggests the involvement of other pathways.

The exact mechanisms through which TNF-α inhibition has its beneficial effects have not been defined. Nevertheless, the critical role of TNF-α in DC development was recently shown in experimental models of arthritis and indicated that TNF-α has potential opposing effects depending on the maturational stage of the DC. Another effect of TNF-α blockade in RA might be the control of chemoattraction of inflammatory cells into the synovial compartment. The decreased expression of chemokines by DC after the inhibition of TNF-α *in vitro* suggests an important role for TNF-α in the control of cell influx *in vivo*. In fact, a critical role for TNF-α in the fine tuning of cell influx was demonstrated recently in synovial sections of patients after the administration of anti-TNF-α [47]. Besides the development of semi-mature DC and a diminished production of chemokines, our current study shows that other mechanisms might explain the beneficial effects of TNF-α inhibition. One of these is the deceased production of IL-6, high levels of which are known to block the suppressive effect of CD4+CD25+ T regulatory cells and skew the differentiation of monocytes towards macrophages [48, 49]. The inhibition of IL-6 secretion may thus potentially restore the function of these regulatory T cells and lead to the development of immature DC designed to restore tolerance. The beneficial results derived from clinical trials aiming for IL-6 blockade emphasise its potential role in RA 9. Although the inhibition of TNF-α sparked a revolution in the treatment of RA, the full inhibition of TNF-α does have side effects like serious infections [50], SLE-like disease [51] and symptoms resembling multiple sclerosis (MS) [52]. Detailed information regarding the actions of TNF-α and its inhibition in the modulation of the immune system would increase our understanding of these issues.

Conclusions

We provide evidence for potential new mechanisms whereby neutralisation of TNF-α might achieve its beneficial effects in clinical practice. These mechanisms include the development of semi-mature DC and decreased chemoattraction following inhibition of chemokine production. Further research into the precise mechanisms of TNF-α on DC mediated T cell polarisation and chemokine production is warranted.

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chapter 3

NOVEL INSIGHTS IN THE REGULATION OF CCL18 SECRETION BY MONOCYTES AND DENDRITIC CELLS VIA CYTOKINES, TOLL-LIKE RECEPTORS AND RHEUMATOID SYNOVIAL FLUID

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Abstract

Background: The T cell attracting chemokine CCL18 is produced by antigen presenting cells and a role for CCL18 has been suggested in the pathogenesis of a variety of diseases. Rheumatoid arthritis (RA) is one of these conditions, in which abundant CCL18 production is present. Although Th2 cytokines and IL-10 are known to have an effect on CCL18 production, there are several gaps in our knowledge regarding the exact regulation of CCL18 secretion, both in general and in RA. In this study we provide new insights in the regulation of CCL18 secretion by monocytes and dendritic cells.

Results: In contrast to a large panel of pro-inflammatory stimuli (IL-1β, TNF-α, IL-10, IL-13, IL-15, IL-17, IL-18, IFN-γ), T cell mimicking molecules (RANKL, CD40L) or TLR driven maturation, the anti-inflammatory IL-10 strongly stimulated DC to secrete CCL18. On freshly isolated monocytes, CCL18 secretion was induced by IL-4 and IL-13, in strong synergy with IL-10. This synergistic effect could already be observed after only 24 hours, indicating that not only macrophages and dendritic cells, but also monocytes secrete CCL18 under these stimulatory conditions. A high CCL18 expression was detected in RA synovial tissue and incubation of monocytes with synovial fluid from RA patients clearly enhanced the effects of IL-4, IL-13 and IL-10. Surprisingly, the effect of synovial fluid was not driven by IL-10 of IL-13, suggesting the presence of another CCL18 inducing factor in synovial fluid.

Conclusion: In summary, IL-10 synergistically induces CCL18 secretion in combination with IL-4 of IL-13 on monocytes and monocyte derived cells. The effects of IL-14, IL-13 and IL-10 are strongly enhanced by synovial fluid. This synergy may contribute to the high CCL18 expression in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is mainly characterized by inflammation of the synovial tissue (ST), leading to cartilage and bone destruction. Influx of different inflammatory cells into the ST and enhanced production of cytokines and chemokines are all well known features of RA. Chemokines are small proteins that act as key players in the chemo-attraction of different leucocytes and perform their chemo-attractive task through interaction with their receptor on the target cell. Several chemokines have been shown to be abundantly present in RA ST at highly strategic sites [1-3], which suggests a role for these chemokines in the pathogenesis of RA. In this respect, chemokines could be regarded as promising therapeutic targets in RA. This concept has already been translated to the clinic, since the blockade of C Chemokine Receptor 1 (CCR1) has recently been shown to be clinically effective in the treatment of RA [4].

Antigen presenting cells (APC), such as dendritic cells (DC) and macrophages (MΦ), are generally accepted as critical mediators in the complex pathogenesis of RA [5-7]. APC produce a multitude of chemokines that attract specific T cell subsets. Such chemokines are likely to play a critical role in the regulation of immune responses, since they orchestrate the spatial and temporal interaction between APC and T cells, which determines the fate and nature of the immune response. Evidence for this conceptual framework came recently from the observation that blocking APC-T cell interactions using CTLA4-Ig led to a significant reduction of disease activity in RA [8]. Several chemokines orchestrate the attraction of T cells toward DC. It is tempting to speculate that interfering with these chemokines would lead to similar effects on disease activity as the direct blockade of T-cell DC interaction. Of this group of T- cell attracting chemokines, CCL18 and CXCL16 recently came out as potentially interesting targets in RA from previous research by our group and others [9-13].

CC chemokine ligand 18 (CCL18, also DC-CK-1, PARC, AMAC-1) was first identified as a naïve T cell attracting chemokine [14-16]. Next to chemo-attraction, CCL18 plays a role in stimulation of collagen production by fibroblasts [17]. Despite numerous attempts to identify its receptor, CCL18 is still an orphan chemokine. *In vivo*, CCL18 expression was first found in high quantities in the lung, which is caused by the abundant expression by alveolar MΦ [15]. *In vitro*, DC and MΦ have been identified as CCL18 producers [14-16, 18, 19]. To date, a substantial amount of data points toward the enrichment of DC and MΦ in the synovial tissue which likely to be responsible for the increased levels of CCL18 in RA synovial tissue and synovial fluid (SF) compared with that from healthy individuals [18, 20]. In this line, CCL18 has been identified as a clinical marker in Gaucher's disease, a condition in which MΦ activation is likely to play a role in the pathogenesis [21, 22]. In addition, a role for CCL18 has been suggested

in a large variety of diseases, such as systemic sclerosis and acute lymphoblastic leukaemia [23, 24]. In RA, we recently found that circulating CCL18 levels are elevated compared with controls and correlated with disease activity [25]. Moreover, CCL18 mRNA expression by DC from RA patients was shown to be higher than by DC from healthy controls, which could be influenced by blockade of TNF-α [10, 13]. The exact regulation of CCL18 protein secretion however is complicated and the studies published thus far have led to controversial results [18, 19, 26-28], as elegantly reviewed by Schutyser *et al* [29].

In order to clarify the mechanism of CCL18 expression and secretion in RA, we investigated the role of a large panel of inflammatory mediators known to play a role in the disease process on CCL18 secretion. Here, we show that CCL18 secretion by monocytes and DC is regulated by synergistic effects between IL-4/IL-13, IL-10 and RA SF, whereas pro-inflammatory cytokines and Toll-like receptor (TLR) ligands did not have any influence on CCL18 secretion. These data add novel information to the puzzle of increased CCL18 expression in RA.

Methods

Patients and samples

For cell culture experiments, 50ml peripheral blood was taken from healthy volunteers and RA patients after receiving informed consent in 10ml lithium heparine (Vacutainer, USA) tubes. Synovial biopsies from RA patients were taken with small needle arthroscopy (Storz, Tutlingen, Germany). Synovial fluid from RA patients was obtained during arthroscopy. For our experiments in which monocytes were stimulated with SF, a pool of SF from 10 different RA patients was used. Synovial samples from healthy controls were taken during scheduled arthroscopic procedures by orthopedic surgeons in patients with traumatic knee injuries. The Nijmegen medical ethics committee (MEC) approved these studies.

Recombinant proteins and antibodies

For stimulation of iDC, we used 20 ng/ml recombinant (rh) IL-1β, rhTNF-α, rhIL-10, rhIL-13, rhIL-15, rhIL-17, rhIL18, 10 ng/ml IFN-γ (all R&D systems, Minneapolis, USA), or 20 ng/ml RANKL and CD40L (Pepro Tech, Rocky Hill, USA). DCs were cultured with 500 U/ml IL-4 and 800 U/ml GM-CSF. The same IL-4 concentration was used for monocyte stimulations. For Toll-like receptor stimulation, 10 μg/ml pam3cys (TLR2), 25 μg/ml poly (i:c) (TLR3), 2 μg/ml lipopolysacharide (LPS) (TLR4), or 1 μg/ml R848 (TLR7/8) was used [30]. Blockade of IL-10 (Ebioscience, San Diego, USA) and blockade of IL-13 (Diaclone, Becanson, France) was achieved with a 1000x excess of a neutralizing antibody. For FACS analysis, we used mouse-anti human antibodies against CD14, (Dako, Glostrup, Denmark), CD83 (Beckman Coul-

ter, Mijdrecht, The Netherlands), IL-4Rα (Santa Cruz, California USA), IL-13RαII (R&D systems, Minneapolis, USA) and IL-10Rα (R&D systems, Minneapolis, USA) or mouse-isotype control (goat IgG for IL-13RαII). For ELISA, mouse anti-human and biotynilated goat anti-human CCL18 were used as capture and detection antibody (R&D systems, Minneapolis, USA). A standard curve was made with rhCCL18 (R&D systems, Minneapolis, USA). Immuno histochemistry for CCL18 was performed with AZN-CK18B [18] as a primary antibody.

Monocyte/macrophage and MoDC isolation and culture

MoDC were cultured using essentially the same protocol as described previously [13, 31]. In brief, peripheral blood mononuclear cells were isolated from venous blood by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Roosendaal, The Netherlands). The interphase was collected and washed with citrated phosphate buffered saline, and the cells were allowed to adhere for 1 hour at 37°C in RPMI-1640 (Life Technologies, Breda, The Netherlands) supplemented with 2% human serum in culture plates (Costar, Badhoeverdorp, The Netherlands). Adherent cells were cultured in RPMI-1640 Dutch modification supplemented with 10% fetal calf serum L-glutamine (Life Technologies, Breda, The Netherlands) and antibiotic-antimycotic agents (Life Technologies, Breda, The Netherlands) (culture medium) in the presence of IL-4 (500 U/ml; Strathmann Biotech, Hamburg Germany) and granulocyte monocyte-colony stimulating factor (GM-CSF) (800 U/ml; R&D systems, Minneapolis USA) for 6 days. Fresh culture medium with the same supplements was added at day 3, and then iDC were harvested at day 6. To generate mature DC, immature DC were re-suspended in a concentration of $0.5x10^6$/ml in fresh IL-4 and GM-CSF containing culture medium. Immature DC were then stimulated with cytokines or maturation stimuli in the presence of IL-4 and GM-CSF. DC were harvested after another 48 hours of culture. For CCL18 measurements in supernatant of cells stimulated with TLR ligands, aliquoted culture supernatant from previous experiments was used 30.

For the culture of monocytes/macrophages, CD14+ cells were isolated with magnetic cell sorting and separation (MACS). In brief, mononuclear cells were labelled with anti CD14 microbeads (Miltenyi Biotec, Amsterdam, the Netherlands) and incubated for 30 minutes at 4°C. CD14 positive cells were then separated from the other cells using a MACS column (Miltenyi Biotec, Amsterdam, the Netherlands) according to the manufacturers instructions. CD14+ cells were cultured in a concentration of $0.5x10^6$ cells/ml in culture medium for up to 6 days. Where appropriate, fresh culture medium was added on day 3. After 6 days, supernatant was collected for ELISA and cells were prepared for FACS analysis. In some additional experiments, monocytes/macrophages were cultured for three days in the same concentration and in the same

media in teflon bags [32] or in rotation discs (Cellon, Luxembourg) [33] to prevent adherence of the cells. In experiments in which monocytes/macrophages were stimulated with RA SF, the cells were cultured for three days in the presence of 100 µl RA SF. Cells were then harvested and re-suspended in fresh culture medium without SF, but with the cytokines that were present in the first three days. Anti-IL-10 or anti-IL-13 neutralizing antibodies were only present during the first three days.

Immuno histochemistry
For immuno histochemistry, frozen ST was cut into 7 µm sections and mounted on slides, air-dried, and stored at -80°C. Before staining, the cryosections were air-dried, fixed in acetone for 10 min and air-dried again. The sections were then stained with 5µg/ml mouse anti human CCL18 or isotype control at 37°C for 1 hour at room temperature (RT) and washed in PBS. Endogenous peroxidase was blocked with 0.3% H2O2/methanol. After another wash-step, the sections were incubated with a biotin-conjugated horse anti-mouse antibody at RT for 30 min. Next, the samples were washed and incubated with avidin-biotin-HRP complex (Vector, Burlingham, UK) at RT for 20 min. Next, the section were stained with diaminodenzidine (DAB) (Sigma, Zwijndrecht, the Netherlands). Finally, sections were then counterstained with hematoxylin, rehydrated and mounted in to allow microscopic evaluation of the samples.

Fluorescence-Activated Cell Sorter (FACS) analysis
The phenotype of cells was characterized by using flow cytometry techniques (FACS). For this aim, cells were harvested and collected by means of centrifugation and further processed on melting ice. Cells were diluted in buffer solution (PBS with 1% bovine albumine, pH 7.4) in a concentration of 110^6 cells/ml and plated in v-shaped 96 wells plates (110^5 cells per plate). Cells were labeled with monoclonal mouse- or goat anti human antibodies or mouse-isotype control (goat IgG for IL-13RαII) and incubated at a temperature of 4°C for 45 minutes. Cells were then washed and labeled with goat-anti-mouse (or rabbit anti-goat when appropriate) FITC (Zymed Laboratories, South San Francisco, USA) as a secondary antibody. After another 30 minute incubation at 4°C, cells were again washed, diluted in buffer solution and transferred into FACS tubes. Cells were gated according to their forward and side scatters and fluorescence was measured with a FACSCalibur® (Becton-Dickinson, San Jose, USA) and Cellquest® software.

Enzyme Linked Immuno Sorbent Assay (ELISA)
For the detection of CCL18 protein levels of CCL18 in supernatant, a sandwich ELISA was performed as described previously [18, 34]. In brief, maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated overnight with 50 µl/well 1 µg/ml capture

antibody in PBS. Next, the plates were washed 3 times with PBS and blocked with 300 µl 1% Bovine Albumin (Sigma, Zwijndrecht, the Netherlands) in PBS for a minimum of 1h at RT. After washing 3 times with ELISA wash buffer (PBS containing 0.05% Tween-20), the plates were incubated with 50 µl/well of serial dilutions of the sample for 2 hrs at RT. Serial dilutions of rhCCL18 were used to obtain a standard curve. After washing 3 times with ELISA wash buffer, the plates were incubated with 50 µl/well of 0.05 µg/ml secondary antibody at RT for 1 hr. Thereafter, the plates were washed 3 times with ELISA wash buffer, and incubated with 50µl /well of streptavidin conjugated to Poly-Horse Radish Peroxidase (CLB, Amsterdam) for 20 minutes at RT. After washing 3 times with ELISA wash buffer, the presence of HRP was detected using 50 µl/well 3,3',5,5-tetramethylbenzidine (TMB) (Biomerieux, Marcy l'Etoile, France) diluted in peroxide buffer (UP) (Biomerieux, Marcy l'Etoile, France). The reaction was stopped with 50 µl/well 2,5M H_2SO_4. Absorbance was measured at 450 nm using a Magellan Tracker V4.XX (Tecan Austria GMBH). As an internal control for inter-assay variability, a sample of pooled normal human serum (n=300) was taken along in all assays. The maximal accepted inter-assay variability is 10%. The detection limit of the ELISA is 100 pg/ml.

Statistical analysis

CCL18 production levels by monocyte derived cells upon different stimulations were compared with a Wilcoxon Signed Rank test. p-values < 0.05 were considered significant.

Results

IL-10 strongly enhances CCL18 production by MoDC while maturation and pro-inflammatory mediators do not.

First we investigated whether several mediators that are known to be important in RA were able to enhance CCL18 production by MoDC. In line with previous studies, unstimulated immature DC (iDC) produced significant amounts of CCL18 [19]. Interestingly, incubation with TNF-α, IL-1β, IL-13, IL-15, IL-17, IL-18 and IFN-γ did not stimulate CCL18 secretion when added to day 6 iDC (n=6). In contrast, the anti-inflammatory IL-10 strongly induced CCL18 production by iDC (p=0.03) (figure 1a). Next we tested whether factors well known to induce maturation or T cell mimicking could induce CCL18 production. These experiments demonstrated that LPS, CD40L and RANKL did not enhance CCL18 production (n=3) (figure 1b). Recent studies demonstrated that other TLR pathways than TLR4 are all capable of inducing DC maturation, but have different effects on cytokine production [30, 35, 36]. However, stimulation of TLR2 (pam3cys), TLR3 (poly (i:c)), TLR4 (LPS) or TLR7/8 (R848) did not sort any effect on CCL18 secretion (n=6) (figure 1c), whereas they did elicit a potent

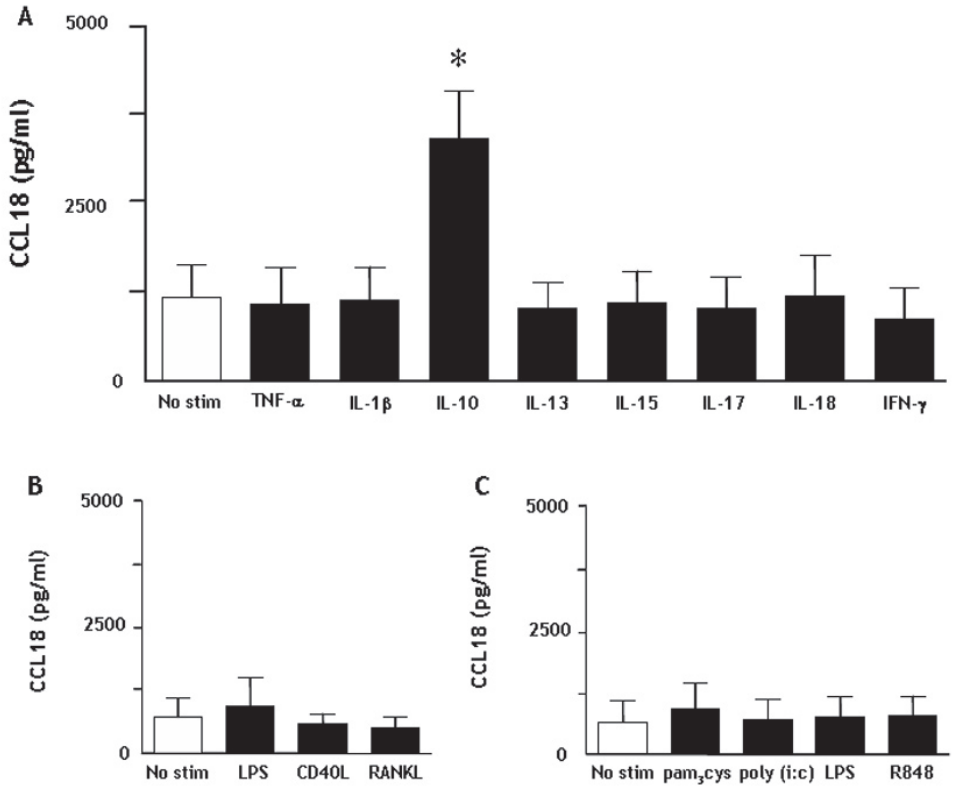


Figure 1.

IL-10 induces CCL18 secretion by monocyte derived dendritic cells.

Panel A depicts CCL18 secretion by MoDC (cultured with IL-4 and GM-CSF) upon stimulation with TNF-alpha , IL-1beta , IL-10 , IL-13 , IL-15 , IL-17 , IL-18 (all 20 ng/ml) and IFN-gamma (10 ng/ml) (n=6). Panel B depicts CCL18 secretion by MoDC upon stimulation with LPS (2µg/ml), CD40L or RANKL (20 ng/ml) (n=3). Panel C depicts CCL18 secretion upon stimulation upon TLR2 (pam3cys, 10 µg/ml), TLR3 (poly (i:c), 25 µg/ml), TLR4 (LPS, 2 µg/ml) or TLR7/8 (R848, 1 µg/ml) mediated stimulation (n=5) In all experiments, a direct comparison was made with unstimulated cells. The bars represent the mean (±SEM) CCL18 secretion in pg/ml .

* represents a p-value of <0,05 (Wilcoxon Signed Rank test)

cytokine response [30]. Since IL-13 is more abundantly present in RA than IL-4 and since some conflicting results have been published on CCL18 production induced by LPS when DC were cultured with IL-13 vs. IL-4, we compared these culture methods (n=6). In both the IL-4 and IL-13 cultures, IL-10 strongly induced CCL18 (p=0.03 for both IL-4 and IL-13 culture), while LPS again did not (figure 2). In addition, IL-10 in combination with LPS was not significantly different from IL-10 alone (figure 2). Also co-stimulation with LPS and the cytokines tested (as in figure 1) did not sort any effect on CCL18 secretion (data not shown).

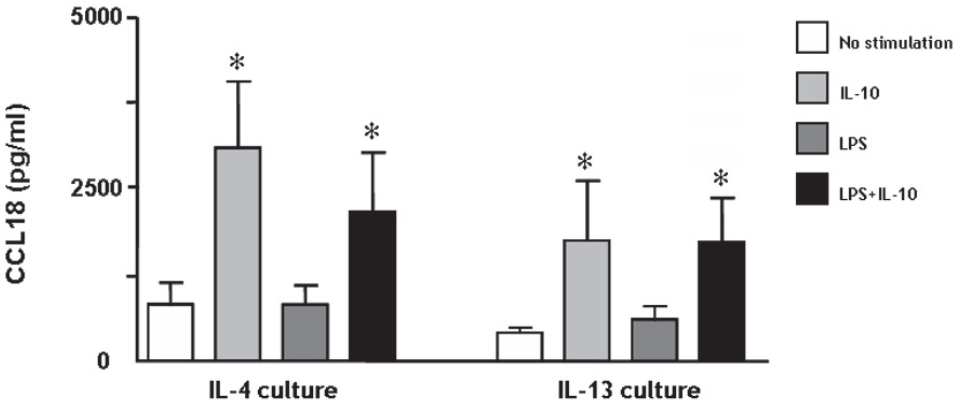


Figure 2.

Similar pattern of CCL18 production by IL-4 vs. IL-13 cultured monocyte derived dendritic cells.

Immature MoDC were initially cultured with either IL-4 or IL-13, in combination with GM-CSF. On day 6, these immature DC were stimulated for 48 hours with IL-10 (20 ng/ml), LPS (2µg/ml) or both. The bars represent the mean (±SEM) CCL18 (pg/ml) production/ml of 6 individual experiments.

* represents a p-value of <0,05 (Wilcoxon Signed Rank test)

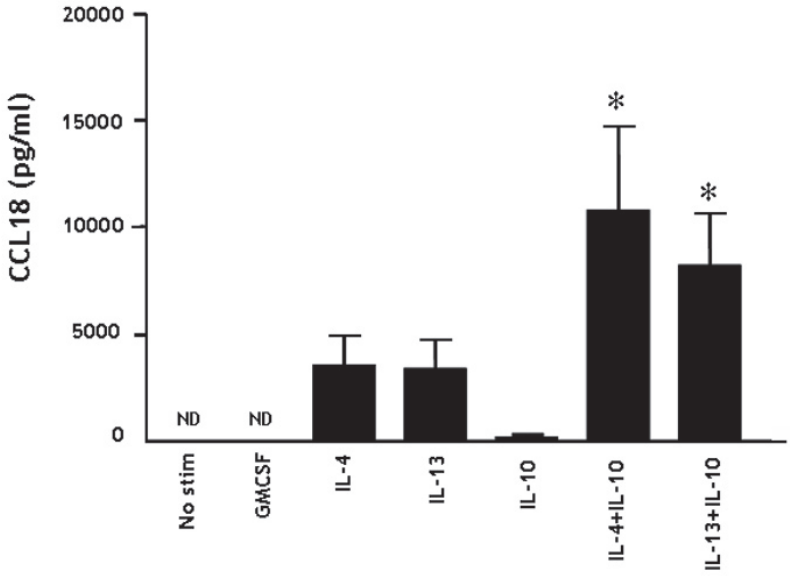
IL-10 acts in synergy with IL-4 /L-13 in promoting CCL18 production by monocytes.
MoDC and alternatively activated MΦ (AaMΦ) [37, 38] are known to produce CCL18. Both these cell types originate from CD14+ monocytes and depend on IL-4 or IL-13 (in combination with GM-CSF for MoDC) for their differentiation. To determine whether CCL18 secretion by myeloid cells is dependent on these cytokines, monocytes were freshly isolated and stimulated with GM-CSF, IL-4, IL-13 and IL-10 alone or in combinations (n=6). Even after 6 days, unstimulated and GM-CSF treated monocytes/macrophages did not secrete CCL18, whereas both IL-4 and IL-13 stimulation resulted in a clear secretion of CCL18, which is in line with previous findings on AaMΦ [16]. Interestingly, stimulation with IL-10 alone only had a minimal effect on CCL18 production by these monocytes/macrophages. When IL-10 was provided together with IL-4 or IL-13, this resulted in 3- and 2-fold increase in CCL18 secretion respectively (figure 3). Interestingly, already in low concentrations, IL-10 had its synergistic effect with IL-4 (figure 4a). In order to rule out effects of adherence, we cultured CD14+ monocytes/macrophages for three days in teflon bags [32] and in rotation discs [33]. The morphology of these cells was comparable with freshly isolated monocytes according to their forward/side scatter pattern (data not shown). In both cultures, IL-4 did still induce CCL18 production in the same way as the cultures in 24-wells plates (figure 4b). As a proof of principle, we next tested whether the synergy between IL-4/IL-13 and IL-10 could already be observed after only 24 hours.

Intriguingly, we could indeed observe a clear CCL18 secretion after 24 hours upon stimulation of freshly isolated monocytes with IL-4/IL-13 and IL-10, whereas stimulation with IL-4, IL-13 or IL-10 alone did only result in a minor or even undetectable CCL18 secretion (n=3) (figure 4c). Since IL-10 appeared to synergize with IL-4 and IL-13, we investigated whether these cytokines could up-regulate each other's receptors, possibly resulting in enhanced signaling. This was not the case; IL-10 did not up regulate either the IL-4/IL-13 common receptor IL-4Rα1 or the specific IL-13Rα2. Furthermore, IL-4 had no effects on IL-10Rα (data not shown).

RA synovial fluid enhances CCL18 secretion independently of IL-10 and IL-13.
We and others demonstrated CCL18 expression in RA ST in the lining and the perivascular regions [10, 20]. In figure 5, we show a high CCL18 expression in RA synovial tissue (figure 5a,b,c), which was preferentially located in both the synovial lining layer and the peri-vascular regions. Intriguingly, CCL18 was also expressed in control synovial tissue, although not as abundant as in RA ST (figure 5d,e,f). Notably, some parts of the sections were even negative for CCL18, which is in sharp contrast with RA. In order to explain the abundant CCL18 expression in RA, we tested whether incubation with RA SF could induce CCL18 production on monocytes/macrophages. Since RA SF itself contains CCL18 [18, 20], we cultured freshly isolated monocytes for 3 days in the presence of SF, washed the cells and cultured on for another 3 days in the absence of RA SF (n=6). Firstly, this pre-incubation with RA SF resulted in marked CCL18 production (mean 676 (±151) pg/ml) (figure 6b). Secondly, culture of freshly isolated monocytes in the presence of RA SF, resulted in a 9- and 10-fold increase in CCL18 secretion upon stimulation with IL4/IL-13 respectively and a 22-fold increase compared with IL-10 alone (figure 6a). Intriguingly, this synergistic effect with IL-4, IL-13 and IL-10 could still be observed after 3 days of culture in the complete absence of RA SF (figure 6b), indicating that the cell does not require a continuous stimulation in order to secrete CCL18. To exclude intrinsic differences between monocytes/macrophages from RA patients and controls may contribute to the effects on CCL18 secretion, we tested whether monocytes/macrophages from RA patients (n=3) responded differently to combinations of IL-4, IL-13, IL-10 and SF. No difference in the CCL18 secretion pattern was observed between monocytes/macrophages of healthy controls and RA patients upon these stimuli (data not shown), ruling out intrinsic differences in monocytes in RA that affect CCL18 secretion.
Since the synergy caused by RA SF appeared similar to the synergy between cytokines we already observed (figure 4), we tested whether IL-10 and/or IL-13, both present in RA SF, were responsible for this phenomenon by blocking these cytokines with neutralizing antibodies. The potency of these antibodies was first tested by determining their ability to inhibit the synergistic CCL18 secretion upon stimulation with a combination of IL-10 and IL-4/IL-13. Addition of anti-IL-10 resulted in a 73 %

Figure 3.

Synergistic effect on CCL18 secretion by monocytes upon stimulation with IL-4/IL-13 in combination with IL-10.
MACS isolated monocytes were cultured for 6 days and stimulated on day 1 with IL-4 (500U/ml), IL-13 (20 ng/ml), IL-10 (20 ng/ml) or a combination of the cytokines. The bars represent the mean (±SEM) CCL18 (pg/ml) of 6 individual experiments. In all experiments, a direct comparison was made with unstimulated cells. ND= not detectable
* represents a p-value of <0,05 (Wilcoxon Signed Rank test)



inhibition of the synergy between IL-4 and IL-10 and anti IL-13 completely abrogated the synergistic effect of IL-13 with IL-10 (figure 6c). Unexpectedly however, blockade of IL-10 or IL-13 in SF in the presence of IL-4 or IL-13 and IL-10 respectively did not inhibit the synergy between these cytokines and SF (figure 6a&b), suggesting that SF contains a yet unidentified factor that triggers CCL18 secretion.

Discussion

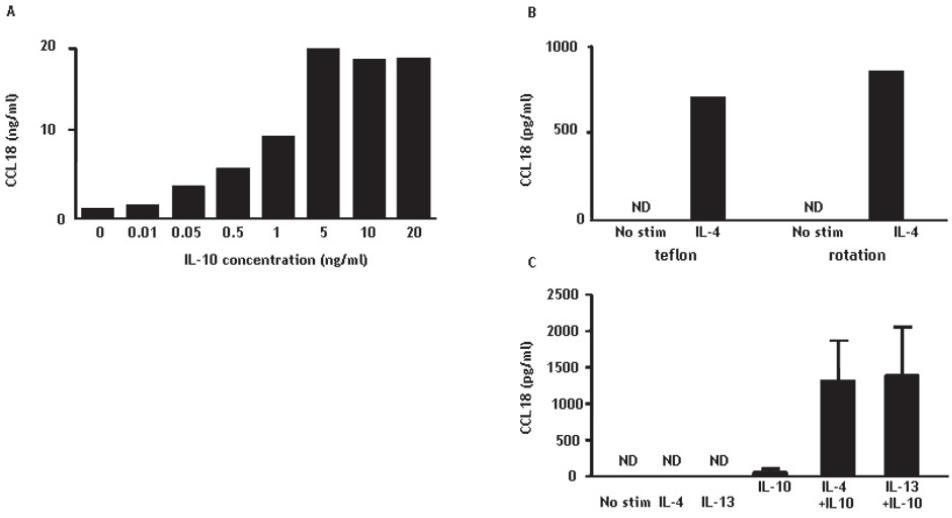
In this study, we add new pieces to the complicated puzzle of CCL18 regulation in RA. Firstly, we demonstrate that CCL18 production can be induced by IL-4, IL-13 and IL-10 in monocyte derived cells. Secondly, we show that a large panel of pro- inflammatory stimuli and TLR mediated signals leading to DC maturation are of no influence on CCL18 production. Thirdly, IL-10 only induces a minor CCL18 secretion, but acts in synergy with both IL-4 and IL-13 on monocytes and monocyte derived cells. Finally, we provide evidence that RA SF is able to induce CCL18 secretion in strong synergy with IL-4, IL-13 and IL-10, which could not be inhibited by a blockade of IL-10 and IL-13.

CCL18 can be produced by MoDC as well as by certain types of MΦ. Often these cell types are considered to be totally different cells. However, the differences between MoDC and AaMΦ are not that large, since monocyte derived macrophages are cultured in the presence of GM-CSF by some groups [39] and both cells require the presence of IL-4 or IL-13. Penna and co workers demonstrated that several in vivo DC subtypes were not able to produce CCL18 [40], which is in contrast with previous findings, where CCL18 mRNA expression was found on CD11c+ myeloid blood DC 28. Moreover, in vitro cultured MoDC have been identified as potent CCL18 producers [18, 19]. These data suggest that a CD14+ monocyte origin in combination with a stimulation by IL-4/IL-13 is critical for CCL18 secretion. This hypothesis is strengthened our data, demonstrating that non-adherent monocytes /macrophages were able to produce CCL18 under the influence of IL-4. In addition, the synergistic effects of IL-4/IL-13 and IL-10 on CCL18 secretion by freshly isolated monocytes were already clearly visible after 24 hours. This indicates that a full differentiation into DC or MΦ is not essential for CCL18 production as has been suggested previously for

Figure 4.

Synergistic CCL18 production by monocytes upon stimulation with IL-4 and IL-10 can be induced rapidly and by low concentrations of IL-10

Panel A depicts CCL18 secretion by monocytes upon stimulation with different doses of IL-10 in the presence of IL-4. Panel B represents CCL18 secretion by monocytes that were cultured for three days in the presence or absence of IL-4 in teflon bags or rotation discs to prevent adherence. Panel C depicts CCL18 secretion by monocytes that were cultured for 24 hours with no stimulation or in the presence of IL-4, IL-13, IL-10 or a combination of IL-4/IL-13 with IL-10. The bars in panel A and B represent the mean CCL18 (pg/ml) of duplicates of 1 individual experiment and panel C shows the mean (±SEM) of 3 separate experiments. ND= not detectable



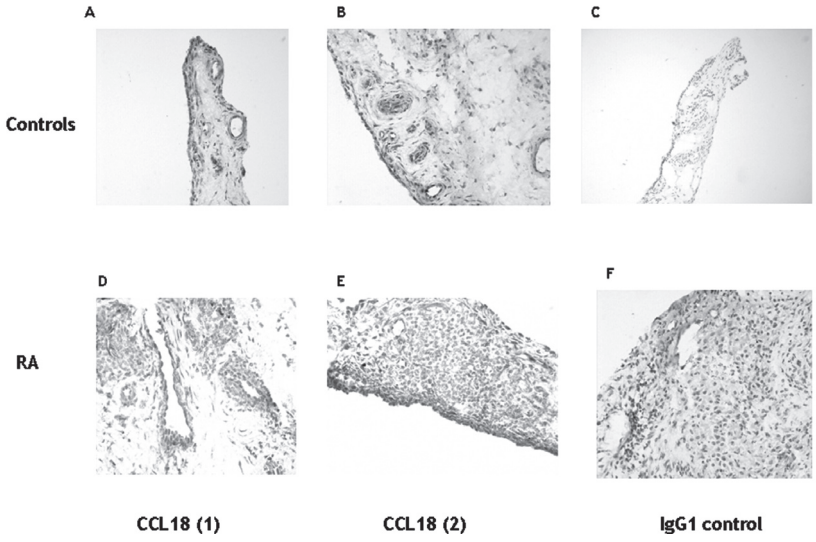


Figure 5

CCL18 expression in normal and RA synovial tissue.

Panel A and B depict 2 sections of control synovium, where CCL18 expression is expressed in parts of the lining and some perivascular regions. Panel D and E depict 2 representative synovial sections from RA where CCL18 is present in the lining and perivascular regions. Panel C and F represent isotype controls on RA synovium and that from healthy individuals respectively.

CCL18 mRNA expression [16]. Thus monocytes rapidly secrete CCL18 upon triggering with the right stimuli.

In the literature there is still some controversy regarding the effect of DC maturation on CCL18 production. Vulcano and co workers suggested that DC down regulate their CCL18 secretion upon maturation [19]. This is in contrast with results from other studies, where maturation caused an increased mRNA expression [10, 27, 28]. A similar contrast between protein and mRNA was found on blood DC [28, 40]. The reason for these differences between mRNA expression and protein secretion patterns still needs to be investigated in detail. Recently, we already provided evidence that DC maturation does not influence CCL18 protein secretion [18], which is further strengthened by the data from the present study, in which different TLR stimulatory pathways did not induce CCL18 production, whereas full DC maturation was achieved [30]. Also TNF-α and CD40L, both well appreciated inducers of DC maturation [41, 42], did not enhance CCL18 production. Perhaps the discrepancy between the different

reports is hidden in subtle differences in culture conditions, which are difficult to trace in the published data. Intriguingly, stimulation with IL-10 alone only lead to a marginal induction of CCL18 secretion by monocytes/macrophages, but did act in a strong synergy with IL-4 or IL-13. The latter is not caused by an up regulation of the receptors IL-10Rα, IL-4Rα or IL-13Rα2 (data not shown). Probably intracellular pathways direct the synergy between these cytokines, which is an interesting topic that warrants further investigation.

We showed that RA SF induces CCL18 production and strongly synergizes with IL-4, IL-13 and IL-10. Blocking studies revealed that neither IL-10 nor IL-13 in SF were responsible for this effect. This suggests the presence of another, yet unidentified CCL18 inducing factor in RA SF. Another explanation for this fact might be the presence of inhibiting factors in SF that counter-regulate the effects of IL-10 and IL-13. The identification of the factor in SF that drives the effects on CCL18 secretion may provide important new insights to the pro- vs. anti inflammatory balance in RA. In order to find this factor in a complex fluid like SF, more knowledge on the pathways of CCL18 regulation is critical. Another intriguing observation from our study is the finding that pre-incubation with SF lead to a sustained synergistic CCL18 secretion upon stimulation with IL-4, IL-13 and IL-10. This could be regarded as an "imprinting effect", meaning that the cell's previous environment determines the nature of response to stimuli, even when the cell is no longer in such an environment. Results from previous studies, in which we showed that MoDC from RA patients differ in phenotype and cytokine response from control DC after 6 days in culture might also be explained by such a phenomenon [31, 43].

Upon the encounter of an antigen, DC normally mature and migrate to lymphoid tissues in order to perform their task of antigen presentation to T cells. Immature DC or MΦ can also encounter naïve T cells in the periphery, which subsequently might result in tolerance [44]. This peripheral tolerance is a critical mechanism to prevent autoimmunity. A role for CCL18 in this part might explain the high expression of CCL18 by alveolar MΦ [15, 16], which are located at a site where the maintenance of tolerance to non-pathogenic antigens, that are constantly present, is crucial. Also the synergistic effect on CCL18 secretion that we found with IL-10, a cytokine that is well appreciated as a pivotal regulator of the immune system, fits in this picture. The synovial lining in the joints has similarities with the alveolar lining in the lung. They both consist of MΦ-like cells and both form a barrier to a site in which self- and non-pathogenic antigens are constantly present. The disease process in RA is considered to be driven by pro inflammatory cytokines such as IL-1β, TNF-α, IL-17 and IL-18 [45-50], whereas CCL18 is regulated by IL-10, IL-4 and IL-13. It is therefore tempting to speculate that

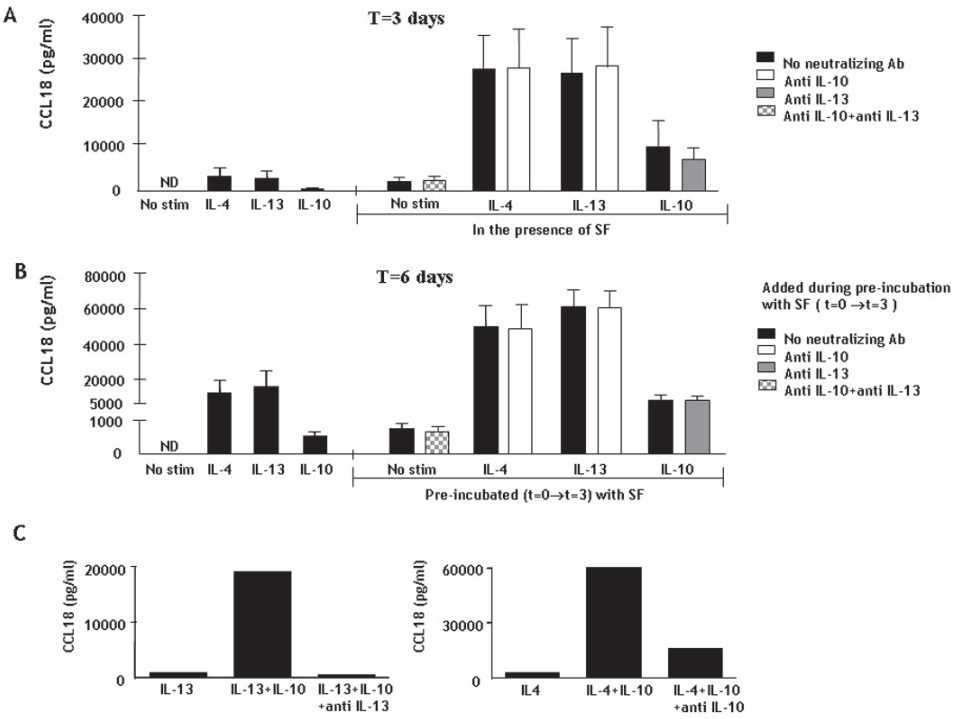


Figure 6

CCL18 production by monocytes upon stimulation with RA synovial fluid

Panel A. Monocytes/macrophages were cultured for three days. A part of the cells were incubated with IL-10, IL-4 or IL-13 alone (left side of the figure) and another part was incubated with these cytokines in the presence of RA SF (right side of the figure). Half of the latter were also incubated with neutralizing antibodies against IL-10 and IL-13 or both, which is shown by the white, gray and checked bars respectively in panel A. The bars represent the mean CCL18 pg/ml from 6 separate experiments. ND= not detectable

Panel B. The cells were then washed and only the cytokines were added again to the fresh medium. After another 3 days, supernatant was measured again. The left side of the figure shows the CCL18 production upon stimulation with IL-4, IL-13 and IL-10. The right side shows the production upon stimulation with these cytokines by cells that have been pre-incubated with SF for three days, in the presence or absence of anti IL-10, anti IL-13 or both (white, gray and checked bars respectively). ND= not detectable

* represents a p-value of <0,05 (Wilcoxon Signed Rank test)

Panel C. The potency of neutralizing antibodies against IL-13 and IL-10 was tested by investigating their ability to inhibit the synergy between IL-13 and IL-10 and IL-4 and IL-10. The bars represent the mean (±SEM) CCL18 secretion (pg/ml).

the high CCL18 expression in RA is designed to uphold peripheral tolerance, which however seems to fail. This failure might be explained in two ways. The first explanation might be that the skewing in the balance towards Th1 is still present despite the upregulation of anti inflammatory mediators. Secondly, mature DC are present in the synovial tissue in perivascular regions and secondary lymphoid organs [3, 51], which is in sharp contrast with healthy synovial tissue. Therefore an explanation for the ongoing immune process might be that these mature DC direct naïve T cells towards a phenotype that drives the pro-inflammatory immune response in the synovial tissue.

Conclusions

In summary, we provide evidence that monocyte derived cells produce CCL18 under the influence of IL-4 and IL-13. IL-10 acts in strong synergy with IL-4 and IL-13 as a key regulator of CCL18 production by monocytes, which indicates that CCL18 secretion is not confined to fully developed DC and MΦ. In addition, the effects of IL-4, IL-13 and IL-10 are strongly enhanced by RASF, which is due to yet unidentified factors. Both the *in vivo* expression pattern and the contributing factors to its regulation *in vitro* are suggestive for a role for CCL18 in the regulation of the immune system, both in health and auto-immune diseases such as RA.

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Chapter 4

ELEVATED CXCL16 EXPRESSION BY SYNOVIAL MACROPHAGES RECRUITS MEMORY T CELLS INTO RHEUMATOID JOINTS

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Abstract

Objective: Directional migration of leukocytes is orchestrated by the regulated expression of chemokine receptors and their ligands. The receptor CXCR6 is abundantly expressed by Th1-polarized effector/memory lymphocytes accumulating at inflammatory sites. This study was undertaken to examine the presence of CXCR6+ T cells and of CXCL16, the only ligand for CXCR6, in the joints of patients with rheumatoid arthritis (RA).

Methods: Flow cytometry analysis was used to examine the expression of CXCR6 by peripheral blood and synovial fluid (SF) T cells. In addition, by performing conventional and real-time RT-PCR, immunohistochemistry and ELISA we determined the expression of CXCL16 and its protease ADAM-10 within synovium and by cultured macrophages. SF T cell migration was studied with the Transwell system.

Results: Accumulation of CXCR6+ T cells within RA SF coincided with highly elevated levels of CXCL16+ macrophages. *In vitro* studies revealed that monocytes start to express CXCL16 upon differentiation into macrophages and that RA SF and tumor necrosis factor (TNF) enhanced CXCL16 expression. Moreover, RA patients responding to anti-TNF therapy show a strongly decreased CXCL16 expression, whereas non-responding patients did not. Interestingly, ADAM-10, a recently identified protease of CXCL16, was abundantly expressed by CXCL16+ macrophages *in vitro* and in RA *in vivo*, resulting in increased levels of cleaved CXCL16 in RA SF relative to controls. Finally, CXCR6+ T cells from RA SF are attracted by CXCL16.

Conclusion: These data provide evidence that enhanced production of CXCL16 in RA synovia leads to recruitment of CXCR6+ memory T cells, thereby contributing to the inflammatory cascade associated with RA pathology.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune which is characterized by a chronic inflammation of multiple synovial joints. Large numbers of leukocytes infiltrate and accumulate within the synovial tissue (ST) and synovial fluid (SF) [1-3]. These leukocytes include T cells, especially CXCR6+ memory T cells, monocytes, plasma cells and granulocytes. While in most patients these cells are dispersed throughout the synovium, in other patients highly organized lymphoid structures resembling germinal centers can be found [4]. Although the cause of RA is still unknown, the recruitment and cytokine-induced activation of inflammatory cells is thought to be essential in perpetuation of the inflammatory response and, ultimately, in cartilage and bone destruction [5-7].

The trafficking of leukocytes is regulated through selective expression of an array of chemokines, adhesion molecules and their corresponding receptors. Chemokines are secreted proteins that attract leukocytes via activation of 7-transmembrane-domain G-protein-coupled receptors [8, 9]. Adhesion molecules provide adhesive capacity during cell-extracellular matrix or cell-cell contact, e.g., when leukocytes transmigrate the endothelium [10, 11]. In this respect CXCL16 is an exceptional chemokine, because it has the potential to function as a chemoattractant and as an adhesion molecule. While classical chemokines are expressed as small soluble proteins, CXCL16 is first synthesized as a transmembrane protein expressed by macrophages (MΦ), dendritic cells (DC) and endothelial cells [12-14]. Data from Shimaoka *et al* 15 have recently suggested that cell-surface-expressed CXCL16 can indeed function as an adhesion molecule. However, upon cleavage by proteases the extracellular domain is released as a soluble chemokine that attracts effector/memory T cells that express CXCR6, the receptor for CXCL16 [12, 14]. Furthermore, CXCL16 also acts as a scavenger receptor for oxidized LDL and bacteria [13, 16, 17], confirming that CXCL16 is a multifunctional protein. Concerning structure and mechanism of action CXCL16 resembles the other transmembrane chemokine: fractalkine. Also fractalkine has been shown to mediate adhesion in its transmembrane form, and to mediate chemotaxis as a cleaved protein [18-20].

Kim and colleagues recently reported the accumulation CXCR6+ T cells in SF of a small number of RA patients [21]. As yet, however, nothing is known about the expression of CXCL16, the only known ligand for CXCR6, in RA joints. Therefore, we analyzed the expression of CXCL16, its recently characterized protease ADAM-10, and CXCR6 *in vitro* and *in vivo* within healthy joints and in the joints of RA patients. Our data demonstrate that expression of CXCL16 and ADAM-10 is strongly enhanced in RA synovial, resulting in the recruitment and accumulation of CXCR6+ memory T cells in RA joints. These data imply an important role for CXCL16-CXCR6 in synovial inflammation that is strongly associated with RA pathogenesis.

Patients and methods

Patients and samples

ST, SF and peripheral blood were obtained from a total of 43 RA patients attending either the Department of Rheumatology or the outpatient clinics of the University Medical Center (UMC) Nijmegen. All patients fulfilled the American College of Rheumatology criteria for RA [22] and gave informed consent for the study. The disease activity was assessed using the disease activity score for 28 joints (DAS28) [23]. For the present study, ST, obtained from RA patients (n=13) scored as having very active RA (DAS28 > 5.1), was compared with that from healthy individuals (n=5). In addition, SF was obtained from 17 RA patients with active disease, 5 osteoarthritis (OA) patients and 2 controls with trauma injury. Therapeutic regimens of all RA patients were recorded before blood sampling. Patients receiving either prednisolone or biologic therapies, such as anti tumor necrosis factor (anti-TNF) or interleukin 1 (IL-1) receptor antagonist, within 6 weeks prior to the study were not included in the current analysis. For immunohistochemical analysis, synovial biopsies from RA patients (n=13) were obtained using small needle arthroscopy. An average of 20 biopsy samples was obtained from the medial and lateral suprapattelar pouch on each occasion. For comparison, ST from healthy controls (n=5) was obtained during arthroscopic procedures performed by orthopedic surgeons. During these procedures, SF was isolated and collected when possible. In addition, we isolated peripheral blood mononuclear cells (PBMC) from a total of 18 healthy individuals. To analyze the effect of TNF blocking therapy, ST was isolated from RA patients (n=6) before and 6 weeks after treatment with the human anti-TNF monoclonal antibody (adalimumab; 40 mg subcutaneously every other week). This study was approved by the Ethics Committee of the UMC Nijmegen.

Isolation of synovial fluid mononuclear cells

Synovial fluid mononuclear cells (SFMC) from resuspended RA SF were obtained by density gradient centrifugation over Lymphoprep (Axis-Shield, Oslo, Norway). SFMC in the interphase were collected, washed extensively with citrated phosphate buffered saline (PBS), and used immediately for fluorescence activated cells sorting (FACS) analyses and/or migration assays.

Generation of monocyte-derived macrophages

To generate MΦ, PBMC were isolated from buffy coats by density gradient centrifugation over Lymphoprep (Axis-Shield). PBMC in the interphase were collected, washed extensively with citrated PBS, and allowed to adhere to plastic for 1 hour. Next, the peripheral blood lymphocytes (PBL) were washed away and the adhering monocytes were differentiated into MΦ by culturing in RPMI 1640 ("Dutch modification") supplemented with L-glutamine and antibiotic-antimycotic (both from

Invitrogen, Breda, The Netherlands) plus 5% human serum (HS) (Sigma, Steinheim, Germany) for up to 10 days. Culture medium was refreshed every 3 days. Analysis by flow cytometry demonstrated that these MΦ express high levels of CD14 and CD11c, intermediate levels of MHC class II and CD86, and no CD80 and CD209/DC-SIGN (data not shown). During some experiments, either freshly isolated monocytes or day 3 MΦ were cultured in the presence of RA SF or recombinant human TNF (PeproTech, London, UK) for 2 days.

RNA isolation, RT-PCR, and real-time PCR

Total RNA was isolated from synovial biopsies or tonsil tissue sections using Trizol (Invitrogen) according to manufacturers description. After treatment with DNase I (Roche Applied Science, Almere, The Netherlands), first-strand cDNA synthesis was performed by a standard reverse transcription reaction, using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) and pd(N)6 random hexamers (Amersham Biosciences, Freiburg, Germany). Synthesis was performed at 20°C for 10 min, 42°C for 45 min, 95°C for 10 min, followed by cooling-down at 4°C. As a negative control, the reaction was also performed in the absence of reverse transcriptase. PCR was performed with AmpliTaq DNA Polymerase (Applied Biosystems, Niewerkerk a/d IJssel, The Netherlands), 100 ng primer, 200 μM dNTPs (both from Amersham Biosciences) and 1.5 mM $MgCl_2$ in PCR Buffer (both from Applied Biosystems). The primers used for CXCL16 amplification were 5'-CCCGCCATCGGTTCAGTTCA-3' and 5'-GTGGACTGCAAGGTGGACAG-3', for ADAM-10 were 5'-CGGAACACGAGAAGCTGTGA-3' and 5'-AAGTCTGTGGTCTGGTAAATT GTATCA-3', and for actin were 5'-GCTACGAGCTGCCTGACGG-3' and 5'-GAGGCC AGGATGGAGCC-3'. PCR was started with a 5 min denaturation step at 95°C, after which amplification was performed in 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 30 sec. After a final elongation step for 10 min at 72°C, samples were cooled to 4°C and analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide.

Quantitative real-time PCR was essentially performed as described previously [24]. Briefly, amplifications were performed with SYBR Green Master Mix on an ABI/PRISM 7000 sequence detector system (both Applied Biosystems). Quantification of the PCR signals was performed by comparing the cycle threshold (Ct) value in duplicate, of the gene of interest of each sample with the Ct values of the reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Furthermore, the housekeeping gene porphobillinogen deaminase (PBGD) was used as an internal control for the amount of cDNA in every individual. The primers (Eurogentec, Maastricht, The Netherlands) used for CXCL16 real-time PCR were 5'-CTTCATTTTTTGCTGA TGGTTCC-3' and 5'-GTCCCAGCACGGCACCT-3', for GAPDH were

5'-GAAGGTGAAGGTCGGAGT-3' and 5'-AGATGGTGATGGGATTTC-3', and for PBGD were 5'-GGCAATGCGGCTGCAA-3' and 5'-GGGTACCCACGCGAATCAC-3'.

Antibodies

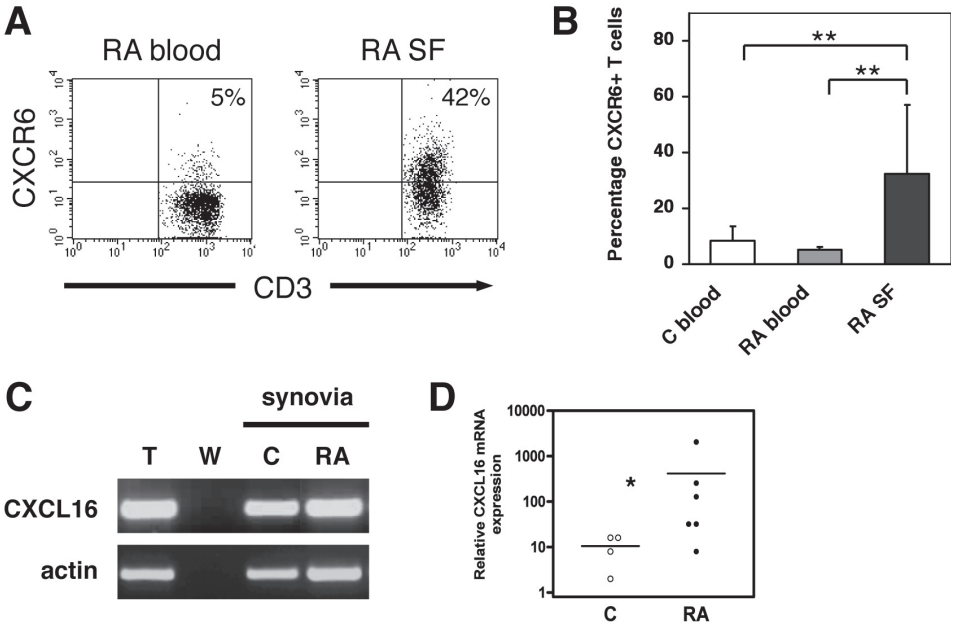
Next to the isotype controls mIgG1, mIgG2a, mIgG2b, (all from BD Biosciences, Alphen aan den Rijn, The Netherlands), rbIgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and gIgG (R&D Systems, Abingdon, UK), the following mAb were used (clone name given in parentheses): anti-human CXCR6 (56811.111) (R&D Systems), PE-conjugated anti-human CD3 (HIT3a) (BD Biosciences), anti-human CD14 (RM052), PE-conjugated anti-human CD14 (RM052), anti-CD208/DC-LAMP (104.G4) (all from Beckman Coulter, Mijdrecht, The Netherlands), anti-human CD31 (JC70A) (DAKO Cytomation, Glostrup, Denmark), anti-human CD45RO (UCHL-1) (BD Biosciences), and anti-human CD68 (EBM11) (DAKO Cytomation). In addition, we used goat anti-human CXCL16 (R&D Systems), biotinylated rabbit anti-human CXCL16 (PeproTech), rabbit anti-human ADAM-10 (Serotec, Oxford, UK), biotinylated horse anti-mouse, biotinylated rabbit anti-rat, biotinylated horse anti-goat; biotinylated goat anti-rabbit (all from Vector, Burlingame, CA), FITC-conjugated goat anti-mouse (Zymed, South San Francisco, CA), PE-conjugated goat anti-rabbit (Caltag Laboratories, Burlingame, CA), AlexaFluor 647-conjugated donkey anti-goat, and AlexaFluor 647-conjugated goat anti-mIgG2b (both from Molecular Probes, Leiden, The Netherlands).

Immunohistochemistry

For immunohistochemistry frozen ST was cut into 7 μm sections, mounted on slides (SuperFrost; Fisher Scientific, Pittsburgh, PA), air-dried, and stored at -80°C. Before staining cryosections were air-dried, fixed in cold acetone for 10 min, air-dried again, and washed with PBS. Endogenous peroxidase was blocked with 1% H_2O_2 plus 0.2% NaN_3 in PBS at room temperature for 10 min. After washing with 0.5% BSA plus 0.01% NaN3 in PBS, sections were then stained with primary Abs at 37°C for 1 hour, followed by incubation with biotin-conjugated secondary Abs at room temperature for 30 min. Next, the samples were incubated with avidin-biotin-HRP complex (Vector) at room temperature for 45 min. Color was then developed with amino ethyl carbazole (Zymed, San Francisco, CA). Sections were counterstained with hematoxylin and mounted in Kaiser's glycerin-gelatin solution (Merck, Darmstadt, Germany). ST sections were analyzed with a DM LB microscope (Leica, Wetzlar, Germany), and photographed using a DC300 camera and Twain Driver-software (IM500) (both from Leica). All immunohistochemical stainings were accompanied by appropriate isotype-matched controls. Per antibody staining at least 2 tissue sections per patient were microscopicaly examined by 2 independent observers who were unaware of the patient's identity and state.

Figure 1

Expression of CXCR6 on T cells from patients with RA. A. FACS results: While a small percentage of peripheral blood T cells from RA patients (RA blood) express CXCR6, this chemokine receptor is widely expressed by RA SF T cells. T cells were stained for CXCR6 and CD3. The quadrants indicate the expression above the background. Dead cells were excluded by gating on propidium iodide-negative cells. Representative examples are shown. B: Percentage CXCR6+ T cells in control blood (C blood) (n=10), RA blood (n=4), and RA SF (n=6). Values are the maean and SD. ** = p<0.01. C: Expression of CXCL16 mRNA in control (C) ST and in synovia from RA patients (RA) was determined by RT-PCR using specific primers. Tonsil mRNA (T) and water (W) were used as positive and negative controls, respectively. Results shown are representative of control synovial and RA synovia. D: Quantification of CXCL16 mRNA demonstrated a significantly enhanced expression in RA synovia (RA, n=6) compared with control synovia (C, n=4). Expression was determined by quantitative real-time PCR. CXCL16 levels were normalized to the levels of the house-keeping gene GAPDH. * = a p<0.05 versus controls.



Flow cytometry

Staining of cell-surface proteins was essentially performed as described previously [25]. Briefly, cells were incubated with the primary Ab at 4°C for 30 min. After washing, the cells were stained with fluorescently-labeled secondary Ab 4°C for 30 min. Propidium iodide was added to exclude dead cells. Flow cytometric analyses were performed on either a FACSCalibur or a FACScan with CellQuest software (all from BD Biosciences).

CXCL16 sandwich ELISA

For the detection of soluble CXCL16 in serum, SF or culture supernatant, a sandwich ELISA was set up. Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated overnight with 50µl/well of 1 µg/ml goat anti-human CXCL16 (R&D Systems) in PBS at 4°C. Next, the plates were washed 3x with PBS and blocked with 100 µl of 1% BSA in PBS for 1 h at 37°C. After washing 3x with PBS containing 0.05% Tween-20 (ELISA buffer), the plates were incubated with serial dilutions of the samples (50 µl / well) for 1 h at 37°C. Serial dilutions of recombinant human CXCL16 (R&D Systems) were used to obtain a standard curve. Samples and recombinant protein were diluted in 1% BSA in PBS. After washing 3x with ELISA buffer, the plates were incubated with 50 µl/well of 0.5 µg/ml biotinylated rabbit anti-human CXCL16 (PeproTech) in ELISA buffer for 30 min at RT. Next, the plates were washed 3x with ELISA buffer, and incubated with 50 µl/well of HRP-conjugated avidin-biotin complex (Vector) in ELISA buffer for 30 min at RT. After washing 3x with ELISA buffer, and 1x with PBS, the presence of HRP was detected using 100 µl/well of 100 µg/ml 3,3',5,5'-tetramethylbenzidine (Sigma) in DMSO (final percentage 1%) diluted in 100 mM NaAc (pH 4.5) buffer. The reaction was stopped with 100 µl/well 800 mM H2SO4. Absorbance was measured at 450 nm using a 3550-UV Microplate Reader (BioRad, Hercules, CA). The detection limit of this ELISA is ~100 pg/ml.

Migration assays

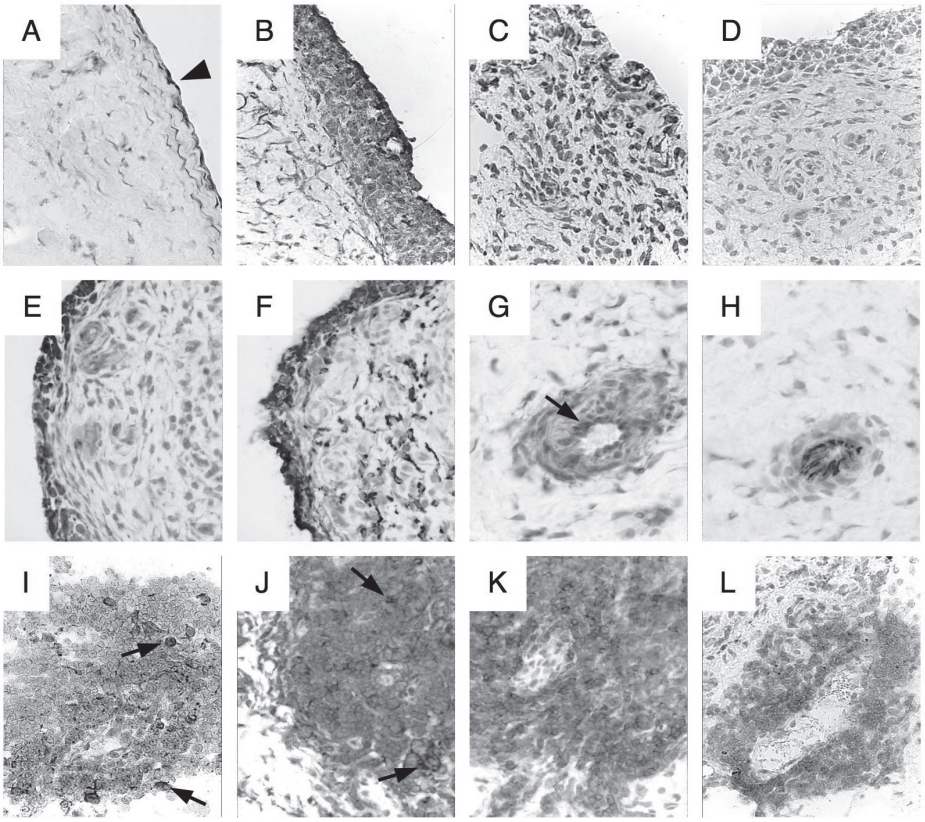
Migratory responses of SF T cells were evaluated by using Transwell polycarbonate inserts (6.5 mm diameter) with 5 µm pores (Costar Corning, Cambridge, MA). SFMC were resuspended in RPMI 1640 (Invitrogen) containing 0.5% BSA (migration medium) and injected (250 X 10^3/100 µl) in the upper compartment of the Transwell. Serial dilutions of recombinant human CXCL16 (R&D Systems) were made in migration medium and added to the lower compartment (600 µl/Transwell). SFMC were allowed to migrate at 37°C in air with 5% CO_2 for 90 min. Next, the inserts were discarded and 20% of the SFMC that had migrated were counted using a flow cytometer as described previously [26]. The remaining SFMC were stained for CD3 to determine the percentage of T cells by flow cytometric analysis.

Statistical analysis

For statistical analyses we first logarithmically transformed the values from groups with a skewed distribution. Next, differences between groups were calculated by using the two sample t test. p-values of less than 0.05 were considered significant.

Figure 2

Enhanced expression of CXCL16 protein within RA synovia. CXCL16 was expressed in the thin synovial lining (arrowhead) of healthy synovia (A), but was much more pronounced by the hyperthrophic lining of RA synovia (B). Many cells present within the sub-lining of RA synovia express high levels of CXCL16 (B and C). Note that (cleaved) CXCL16 was also associated with filaments of the extracellular matrix (B). Staining of serial sections indicated that expression of CXCL16 (E) correlated with the presence of CD68+ synovial macrophages (F). We also detected CXCL16 within some vessels (G). Staining serial sections for CD31 confirmed that these cells were endothelial cells (H). In addition, many lymphocyte aggregates contain CXCL16+ cells (I). Analysis of stained serial sections suggested that these CXCL16+ cells were CD68+ macrophages (J) amidst CD45RO+ memory lymphocytes (K). Cryo-sections were stained for CXCL16 (A-C, E, G and I), CD68 (F and J), CD31 (H), or CD45RO (K), or matched control antibodies (D and I). All sections were counterstained with hematoxylin. Sections shown are representative for ST from 10 RA patients and 5 controls. (Original magnifications x 400 in A-F, and I-L; x 630 in G and H).



Results

SF from RA patients contains increased numbers of CXCR6+ memory T cells

Recent data demonstrated that the chemokine receptor CXCR6 is preferentially expressed by T effector/memory cells involved in Th1 responses and that CXCR6+ T

cells were enriched at sites of inflammation [21]. Therefore, we analyzed CXCR6 expression on T cells isolated from blood and SF of RA patients and healthy individuals. As shown in figure 1a and 1b, both control PBMC (mean 8%) and RA PBMC (mean 5%) contain only small numbers of CXCR6+ T cells. In contrast, up to 80% of the T cells within RA SF expressed CXCR6 (mean 32%), whereas little or no leukocytes could be detected in SF of controls (figure 1a and b, and data not shown). RT-PCR analysis further confirmed that CXCR6 mRNA is indeed expressed by RA SF cells (data not shown). In two patients analyzed, the CXCR6+ T cells displayed a memory phenotype (CD3+CD45RO+) and about 52% of them was CD4+ and 48% CD8+ (data not shown). These results thus confirm and extend the data reported by Kim et al. [21], and demonstrate that high numbers of CXCR6+ T cells specifically accumulate in SF of RA patients.

Increased CXCL16 expression by RA synovial macrophages and endothelial cells
As yet, nothing is known about the expression of CXCL16, CXCR6's only known ligand, in ST or SF. Therefore, we first determined the expression of CXCL16 mRNA in RA ST or control ST by RT-PCR. RNA from a tonsil was included as a positive control. As shown in figure 1c, a CXCL16 PCR product of the expected size was readily detected in both control synovia (n=3) and RA synovia (n=7), as well as in tonsil. Subsequent quantitative analysis demonstrated that CXCL16 RNA levels were significantly increased in RA synovia (n=6) as compared to control ST (n=4) (figure 1d).

Next, we analyzed the expression of CXCL16 in situ by applying immunohistochemistry on tissue sections from 10 RA and 5 control synovia. The results demonstrated that in control synovia the expression of CXCL16 was confined to the single layer of synovial lining MΦ (figure 2a). Synovial tissue from RA patients, however, showed a strong increase in the number of CXCL16+ cells that was most predominant in the thickened synovial lining and sub-lining (figure 2b and c). The number of CXCL16-expressing cells was directly related to the degree of cellularity of the ST (data not shown). The overlapping expression patterns of CXCL16 and the MΦ marker CD68 confirmed that the CXCL16+ cells in the synovial lining were CD68+ MΦ (figure 2e and f). Interestingly, while essentially all blood vessels in control synovia appeared CXCL16 negative (data not shown), some CD31+ blood vessels in RA-synovium expressed significant levels of CXCL16 (figure 2g and h). In addition, CXCL16-expressing cells were also detected within perivascular lymphoid aggregates (Figure 2i). Staining of serial sections suggested that also the CXCL16+ cells that are surrounded by CD45RO+ memory lymphocytes are CD68+ MΦ (figure 2i-k). These results demonstrate that, due to increased numbers of MΦ and activated endothelial cells, the expression of the chemokine CXCL16 is highly increased in RA synovia compared with control synovia.

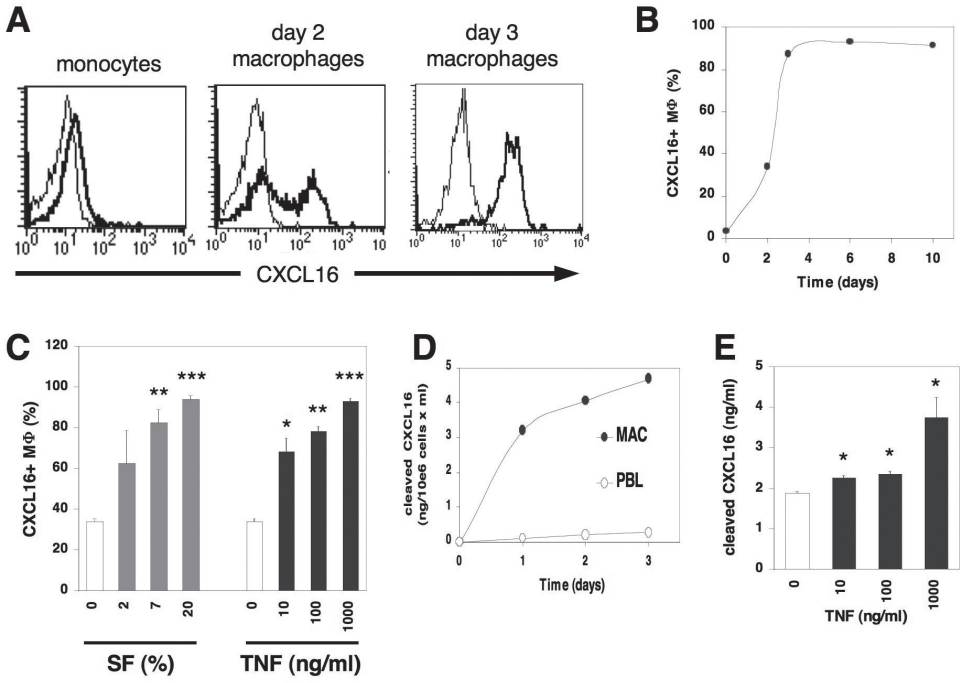


Figure 3

Strongly increased expression of CXCL16 by macrophages after exposure to RA SF or TNF. A: Peripheral blood monocytes were differentiated into macrophages and analyzed by flow cytometry. CD14+ monocytes/ macrophages were gated and stained with anti-CXCL16 (bold lines) or isotype-matched control antibodies (thin lines). Dead cells were excluded by gating on propidium iodide-negative cells. B: Diagram showing the mean percentages of CXCL16+ macrophages at the time points indicated. C: Increasing levels of transmembrane CXCL16 on macrophages, with addition of increasing concentrations of RA SF or TNF to freshly isolated monocytes. At day 2, the percentage of CXCL16+CD14+ macrophages was determined by flow cytometry. The mean and SD percentages from 2 donors are shown. Dead cells were excluded by gating on propidium iodide-negative cells. D: Significant levels of cleaved CXCL16 released by cultured macrophages. By sandwich ELISA the supernatants of macrophages (MAC) or peripheral blood lymphocytes (PBL) cultured for 1, 2 or 3 days were analyzed for the presence of cleaved CXCL16. E: TNF induced expression of cleaved CXCL16. Culture supernatants of day 2 macrophages were analyzed by sandwich ELISA. Representative results out of 2-5 experiments are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus the unstimulated group. See figure 1 for other definitions.

Enhancement of CXC16 expression in macrophages by addition of RA SF or TNF

Next, we investigated the expression of CXCL16 by blood-derived monocytes and MΦ. Whereas freshly isolated monocytes did not express CXCL16 on their cell-surface, upon differentiation into MΦ, they rapidly began to express transmembrane CXCL16 and remained CXCL16+ for at least 10 days of culture (figure 3a and b). Intriguingly, addition of RA SF to freshly isolated monocytes resulted in a significant

and concentration-dependent increase in cell-surface-expressed CXCL16 (figure 3c). Also TNF, one of the constituents of RA SF and a key protein in RA, was sufficient to increase the expression of transmembrane CXCL16.

Because cleavage of CXCL16 is required to exert its chemotactic activity towards CXCR6-expressing lymphocytes, we also analyzed the MΦ culture supernatants for the presence of cleaved CXCL16. As shown in figure 3d, while PBL cultures were essentially negative, significant amounts of cleaved CXCL16 were readily detected in the culture supernatant of day 1-3 MΦ. Moreover, mimicking the RA inflammatory environment through the addition of TNF, further significantly increased the amount of cleaved CXCL16 in the culture supernatant (figure 3e). Because RA SF contains cleaved CXCL16 itself (see below) we did not measure soluble CXCL16 in the supernatant of RA SF-treated MΦ.
To investigate the effect of TNF on the expression of CXCL16 *in vivo*, we determined the CXCL16 expression in synovia of 3 RA patients who responded to the treatment and 3 non-responders. The synovial lining and sub-lining of all patients contained large amounts of CXCL16+ synovial MΦ within prior to treatment (figure 4a and c). Intriguingly, CXCL16 expression was severely reduced in the clinically responding patients (compare figure 4a with 4b) but not in the non-responding patients (compare figure 4c with 4d). Taken together, these data demonstrate that TNF and RA SF, stimuli associated with synovial inflammation, increase the expression of CXCL16 *in vitro* and *in vivo*.

ADAM-10 expression in RA synovia coincides with the presence of cleaved CXCL16 in RA SF.
ADAM-10 has very recently been implicated in the cleavage of CXCL16 [27, 28]. Analysis of monocytes and monocyte-derived MΦ revealed that transmembrane ADAM-10, like CXCL16, was induced on day 2 MΦ and is further upregulated by addition of RA SF (figure 5a and b). Moreover, analysis of serial sections from RA ST showed that expression of the protease ADAM-10 overlapped completely with the CXCL16-expressing MΦ in the hypercellular synovial lining (figure 5f and g). RT-PCR analysis further confirmed that ADAM-10 mRNA was expressed by RA ST (data not shown). In control ST, ADAM-10 staining was limited to the thin synovial lining but still overlapped with the CXCL16 staining (figure 5c and d).

To investigate whether the enhanced expression of both CXCL16 and ADAM-10 by synovial MΦ of RA patients results in elevated levels of cleaved CXCL16, we performed ELISA on serum and SF from controls or patients with either OA or RA. Although cleaved CXCL16 was readily detectable in serum, we did not observe significant differences between sera from RA patients and healthy controls (data not shown). In contrast, analysis of SF demonstrated that RA SF contains significantly

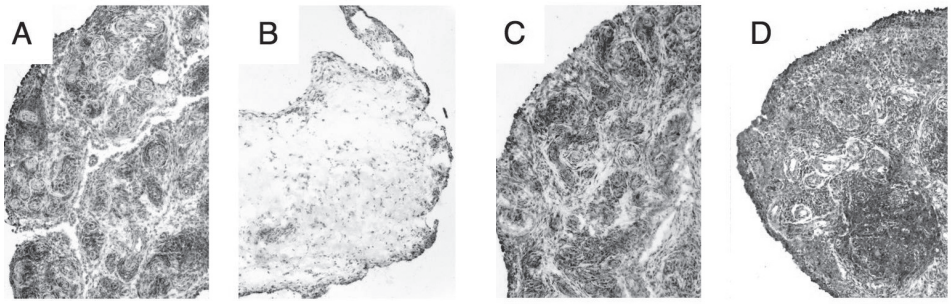


Figure 4

Reduced CXCL16 expression upon successful anti-TNF treatment of RA patients. Before anti-TNF treatment, CXCL16 is strongly expressed within RA synovia (A and C). Expression of CXCL16 is severely reduced in clinically responding patients (B), but not in non-responding patients (D). Synovia were isolated before (A and C) and after (B and D) anti-TNF treatment. All cryo-sections were stained for CXCL16 and counterstained with hematoxylin. Control stainings were negative. Results are representative of 3 responding and 3 non-responding patients. (Original magnifications x 100).

more cleaved CXCL16 than SF from controls (figure 6a). Interestingly, also SF from patients with the milder inflammatory disease OA contained less cleaved CXCL16 than RA SF, although this difference was not significant. These data strongly suggest that the increase in CXCL16 and ADAM10 expressing MΦ in RA ST also results in the release of high amounts of cleaved CXCL16 into the SF in vivo.

Cleaved CXCL16 attracts memory T cells from RA SF

Finally, we determined whether the large numbers of CXCR6+ T cells observed in RA SF were indeed functionally capable of responding to cleaved CXCL16. As shown in figure 6b, RA SF derived T cells rapidly lose their CXCR6 expression upon incubation with increasing concentrations of cleaved CXCL16. These data indicate that the CXCR6+ T cells derived from RA SF internalized CXCR6 upon binding of cleaved CXCL16 and thus are able to respond to CXCL16. Subsequent migration experiments further confirmed that cleaved CXCL16 attracted RA SF T cells in a concentration dependent manner (figure 6c). These results indicate that large numbers of RA SF T cells express functional CXCR6 allowing migration towards strong CXCL16 sources like RA SF.

Discussion

Influx of leukocytes, including CXCR6+ leukocytes, into both ST and SF contributes to the pathogenesis of RA. Here, we report that the chemokine CXCL16, the ligand for CXCR6, is normally expressed by MΦ in the thin lining of healthy synovia. In

RA synovia, CXCL16 expression is elevated and strongly increased due to the presence of large numbers of synovial MΦ. In addition, we show that these CXCL16+ MΦ strongly express the recently identified CXCL16 protease ADAM-10 *in situ*. *In vitro* studies demonstrated that monocyte-derived MΦ express both transmembrane and cleaved CXCL16 and that the expression is enhanced by RA SF and the pro-inflammatory stimulus TNF. Moreover, only successful anti-TNF therapy is associated with decreased CXCL16 expression *in situ*. Finally, elevated expression of both CXCL16 and ADAM-10 by RA ST MΦ *in situ* is associated with high amounts of cleaved CXCL16 in RA SF and with the presence of significantly increased numbers of CXCR6+ T cells in this SF.

CXCL16 is a recently identified transmembrane chemokine expressed by MΦ and DC [12-14]. Upon proteolytic cleavage, the NH2-terminal part of CXCL16 is released and functions as a soluble chemoattractant for CXCR6+ T cells and plasma cells [12, 14]. Interestingly, Kim *et al* reported that CXCR6+ is a marker for effector/memory T cells and that large numbers of CXCR6+ T cells were detected in SF from 3 RA patients [21]. Here, we extended these observations and added novel data concerning the expression and function of CXCR6's only ligand CXCL16. First, we demonstrated that fresh control or RA PBMC contain only small numbers of CXCR6+ T cells (figure 1a and b). In contrast, up to 80% of the T cells within RA SF expressed CXCR6. Immunohistochemical staining of RA ST for CXCR6 revealed no significant staining of T cells (data not shown). However, PCR analysis demonstrated that low levels of CXCR6 mRNA are also present within ST from RA patients (data not shown). Since chemokine receptors are generally expressed at relatively low levels, and we demonstrated that CXCR6 is rapidly down regulated upon CXCL16 binding (figure 6b), these data suggest that CXCR6 is difficult to detect by immunohistochemistry.

Quantitative analysis demonstrated that the expression of CXCL16 mRNA is increased in RA ST as compared to control ST (figure 1d). Immunostaining revealed that in healthy individuals, CXCL16 is expressed by the single layer of cells that make up the synovial lining (figure 2). In RA patients however, elevated expression of CXCL16 is detected in the hypercellular synovial lining, while additional CXCL16+ cells are now detected in the sub-lining and within perivascular lymphocyte aggregates. Overall, CXCL16 expression correlated with increased cellularity of the RA synovia. Based on CD68/CXCL16 staining of serial sections and morphology, the CXCL16+ cells in the lining, sub-lining and lymphocyte aggregates mainly represent MΦ. Although we did not observe co-localization of CXCL16 with the DC-marker CD208/DC-LAMP (data not shown), we cannot exclude that some DC or follicular dendritic cells also express CXCL16. Finally, CD31/CXCL16 staining of serial sections implied that some endothelial cells express CXCL16 (figure 2g and h). These data are in line

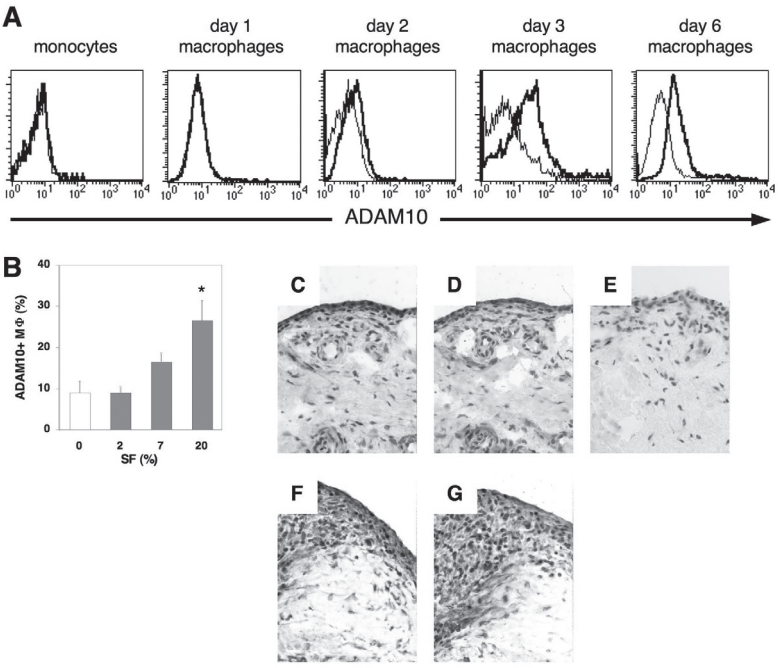


Figure 5

Enhanced expression of CXCL16 protease ADAM-10 by RA synovial macrophages. ADAM-10 negative peripheral blood monocytes were differentiated into macrophages and analyzed by flow cytometry. A: CD14+ monocytes/ macrophages were gated and stained with anti-ADAM-10 (bold lines) or isotype-matched control antibodies (thin lines). Dead cells were excluded by gating on propidium iodide-negative cells. Addition of RA SF to macrophages (day 3) increases their expression of ADAM-10 (B). After 2 days the percentage of ADAM-10+CD14+ macrophages was determined by flow cytometry. The mean and SD percentages from 2 donors are shown. Dead cells were excluded by gating on propidium iodide-negative cells. * = $p<0.05$ versus controls. ST from controls showed expression of both CXCL16 (C) and its protease ADAM10 (D) in the thin synovial lining. In contrast, expression of both CXCL16 (F) and ADAM10 (G) was highly enhanced in the hypercellular synovial lining of RA ST. Frozen sections were stained for CXCL16 (C and F), ADAM-10 (D and G), or a matched control antibody (E), and counterstained with hematoxylin. Representative stainings are shown. See figure 1 for other definitions (Original magnifications x 400).

with previous results demonstrating that cardiac and umbilical endothelial cells can express CXCL16 [29, 30].

Furthermore, our *in vitro* studies show that a significantly larger population of MΦ expressed transmembrane CXCL16 upon addition of RA SF, or TNF, one of the major cytokines in RA SF, than controls (figure 3). Moreover, TNF-treated MΦ also released increased amounts of cleaved CXCL16. This could be of significant importance, since TNF is strongly expressed in RA joints and is considered to be a key player in RA

pathogenesis [31]. In fact, anti-TNF treatment is effectively used in clinics worldwide to treat RA [32-34]. Interestingly, we observed a severely reduced CXCL16 expression in synovial tissue from RA patients responding to anti-TNF treatment, but not in non-responding patients (figure 4). These data suggest that not only *in vitro*, but also *in vivo* the expression of CXCL16 is controlled by TNF. We are currently extending these studies with a larger cohort of RA patients receiving anti-TNF therapy.

Interestingly, highly elevated levels of cleaved CXCL16 were present in SF from RA patients, and this coincided with large numbers of ADAM-10+ MΦ (Figures 5 and 6). ADAM-10 has recently been described to be a major protease involved in the cleavage and release of CXCL16 [27, 28]. Therefore, our data suggest that in RA, ADAM10 expression by the thick layer of synovial lining MΦ is involved in the release of large amounts cleaved CXCL16 in the SF. We note that also significant amounts of CXCL16 are detected in serum of healthy individuals, suggesting that cleavage of CXCL16 also occurs in steady-state conditions. This reasoning is in accordance with our observation that the synovial lining of healthy individuals does express low levels of both CXCL16 and ADAM-10. Since CXCL16 has been suggested to contain multiple restriction sites for proteases [12, 14], also other proteases, e.g. matrix metalloproteinases (MMP) or TNF converting enzyme (TACE), are likely to be involved in the cleavage of CXCL16. With respect to cleavage of CXCL16 in synovium, MMP-1 could be an interesting candidate since we have recently shown that this protease is abundantly expressed by RA synovial MΦ [35, 36].

Finally, we demonstrate that cleaved CXCL16 indeed activates CXCR6 expressed by RA SF T cells (figure 6). First, addition of cleaved CXCL16 to these T cells leads to the loss of cell-surface CXCR6 suggesting CXCL16-mediated CXCR6 internalization. Ligand-induced activation and subsequent internalization is a common feature of chemokine receptors [37]. After being internalized some chemokine receptors recycle back to the cell membrane while others are degraded in the lysosomal compartment. As yet, it is not known how CXCR6 behaves after being internalized. However, we do demonstrate that CXCR6+ T cells isolated from RA SF are capable to migrate in response to CXCL16 *in vitro* (figure 6c). Therefore, our data imply that CXCL16 and CXCR6 play an important role in the recruitment of activated T cells into RA joints.

Several mouse studies have confirmed the importance of chemokines in RA development *in vivo*. Administration of a CCL2/MCP-1 antagonist prevented the onset of arthritis in the MRL-LPS arthritis model [38] and neutralizing CXCL10/IP-10 antibodies prevented adjuvant-induced arthritis [39]. Also in RA patients, adhesion molecules and chemokines play important roles in synovial infiltration and RA pathogenesis. For instance, enhanced expression of adhesion molecules, e.g. E-selectin, VCAM-1 and

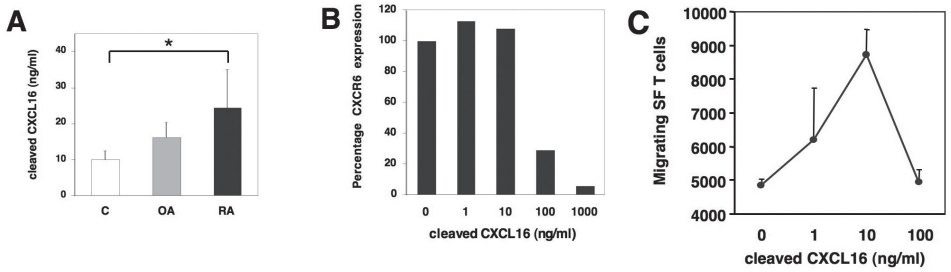


Figure 6

Cleaved CXCL16 is overexpression in RA synovial fluid and attraction of SF T cells. A: Expression of cleaved CXCL16 in RA SF (n=17) was compared with that in SF from controls (n=2), or from patients with OA (n=5). CXCL16 concentrations were measured by sandwich ELISA. Values are the mean and SD. * = $p < 0.02$. B: Loss of cell-surface-expressed CXCR6 on RA SF T cells induced by activation by cleaved CXCL16. The level of CXCR6 expressed by unstimulated T cells was set at 100%. C: SF T cells from RA patients are attracted by soluble CXCL16. Migration was concentration-dependent and reached an optimum at 10 ng/ml. Migration of RA SF T cells was determined in a Transwell migration assay. Migrating cells were counted and stained for CD3 and CXCR6. Representative results from 3 experiments are shown. Values are the mean and SD. See figure 1 for other definitions.

ICAM-1 [33, 40], and various chemokines, including CCL2/MCP-1, CCL5/RANTES, CCL18/DC-CK1 and CXCL8/IL-8, have been detected in RA tissue and/or synovial fluid [41-48]. Interestingly, therapy with a CCR1 antagonist has recently been shown to be beneficial in RA [49]. Oral administration of this antagonist significantly reduced the number of ST macrophages and T cells, and this was correlated with a trend toward clinical improvement as compared to placebo-treated controls. Despite the apparent redundancy in the chemokine system, evidence is accumulating that chemokine and chemokine receptor antagonists have strong potential as therapeutic agents for patients with autoimmune disease [37, 50]. Our data suggest that the use of either CXCR6 antagonists or protease inhibitors acting on CXCL16 cleavage could be additional novel approaches to treat patients with RA.

Based on the results of this study, the following model for the role of CXCL16 – CXCR6 in RA pathogenesis can be envisaged. During inflammation of the joint, locally activated endothelial cells express increased levels of adhesion molecules and chemokines, resulting in enhanced immigration of monocytes. Within the synovial tissue these monocytes now become attracted by chemokines released by the synovial lining, and differentiate into MΦ. Differentiating MΦ start to express both CXCL16 and ADAM-10, expression of which is further enhanced by SF from the synovial cavity, and/or by TNF released by the MΦ themselves. At the now thickened syno-

vial lining, ADAM-10 cleaves transmembrane CXCL16 resulting in elevated concentrations of cleaved CXCL16 in the SF. Cleaved CXCL16 attracts large numbers of CXCR6+ memory T cells into the RA joint. These memory T cells release cytokines like TNF that can now activate MΦ and other resident cells, thus sustaining the inflammatory cascade contributing to RA pathogenesis.

In conclusion, our data suggest that over-expression of CXCL16 targets CXCR6+ memory T cells to synovia from RA patients. Therefore, CXCL16 and CXCR6 could be intrinsically involved in the inflammation associated with RA pathology.

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chapter 5

REGULATION OF CXCL16 EXPRESSION AND SECRETION BY MYELOID CELLS IS NOT ALTERED IN RHEUMATOID ARTHRITIS

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Abtract

Objective: The chemokine CXCL16 is secreted by macrophages and dendritic cells to attract memory type T cells. CXCL16 expression is increased in arthritic joints of patients with rheumatoid arthritis (RA) and a role for CXCL16 has been suggested in the pathogenesis of RA. To date, little is known about the regulation of CXCL16 on monocytes/macrophages and DC. The aim of this study was to elucidate how CXCL16 expression is regulated in healthy donors and patients with RA.

Methods: CD14+ cells were isolated from the peripheral blood or synovial fluid of RA patients and healthy controls, differentiated into different types of dendritic cells or macrophages and stimulated with various cytokines or LPS. Cell surface proteins, including surface CXCL16, were measured by flowcytometry and soluble CXCL16 was measured by ELISA.

Results: Distinct types of dendritic cells constitutively express and secrete CXCL16, which is not affected by maturation. Monocytes rapidly upregulate membrane-bound CXCL16 expression and release soluble CXCL16 upon culture. CXCL16 expression by monocytes is transiently inhibited by the TLR4 ligand LPS. Th2 type cytokines inhibit soluble CXCL16, whereas Th1 stimuli enhance its release. In RA monocytes/macrophages, neither CXCL16 expression, nor CXCL16 regulation is different from healthy controls.

Conclusions: Culture of monocytes is the main trigger for CXCL16 surface expression in vitro, which is not altered in RA. Together our data suggest that the increased CXCL16 expression in patients with RA is likely to be caused by increased influx of monocytes rather than intrinsic differences in CXCL16 regulation.

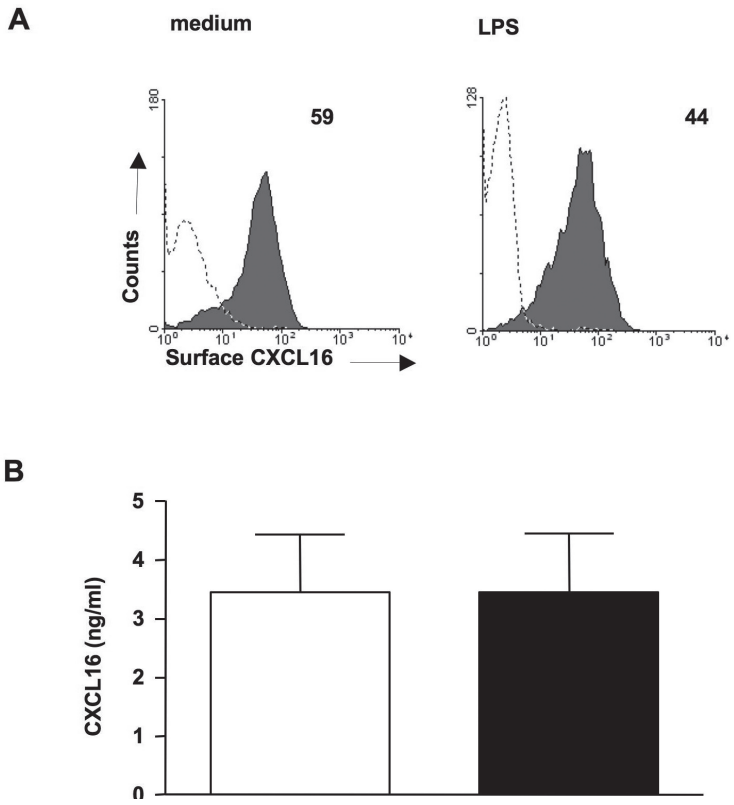

Figure 1:

CXCL16 expression and secretion by MoDC

Panel A shows surface expression of CXCL16 (shaded) vs. goat IgG (dotted) for immature DC (left side) and mature DC matured with 2 µg/ml LPS (right side) at day 8. One representative figure of 10 individual experiments is shown. The accompanying Mean Fluorescence Intensity (MFI) is shown in de figure. Panel B shows the mean (±SEM) CXCL16 secretion of 6 individual cultures upon maturation with LPS for 48 hours. The mean (±SEM) CXCL16 secretion of 6 individual experiments is shown.

Introduction

Rheumatoid arthritis (RA) is a chronic auto-immune disease, characterized by inflammation of the joints, which eventually leads to cartilage and bone destruction. One of the hallmarks of the inflammatory process in RA is the migration of a variety of leukocytes towards the synovial tissue (ST). Among these leukocytes, both antigen presenting cells (APC) and T cells are regarded as important players in RA [1-3]. T cells constitute a large group of lymphocytes that can be divided in an ever growing number of subsets. To date, it is still unclear which exact T cell subsets are most im-

portant in RA. However, the abundance of CD45RO+ lymphocytes in RA synovial fluid (SF) [4] suggests that activated, memory type T cells might play a significant role in RA. The nature of T cell mediated immune responses is largely orchestrated by their interaction with APC, such as dendritic cells (DC) and macrophages. Recently, we and others demonstrated the presence of DC, the most potent APC, in RA synovial tissue [5-7]. Interestingly, DC derived from RA patients have an altered behaviour and phenotype [6, 8, 9], indicating a potential role in RA. The fact that the APC-T cell interaction is nowadays successfully being targeted in a RA patients with CTLA-4Ig [10] strengthens the hypothesis that both DC and T cells play are key players in RA.

In order to attract and interact with other leukocytes, APC produce a variety of chemokines. Chemokines are small secreted proteins which act as chemotactic ligands through interaction with 7-membrane spanning chemokine receptors. CXC chemokine Ligand 16 (CXCL16) is a chemokine that exhibits chemotactic properties towards leukocytes expressing CXC chemokine receptor 6 (CXCR6). This receptor is predominantly expressed on activated, memory type T cells [11, 12], but expression has also been demonstrated on NKT cells and plasma cells [13, 14]. Similar to fractalkine, but in contrast to all other chemokines, CXCL16 exists as a trans-membrane protein and a soluble chemo-attractant. CXCL16 is expressed on the surface of macrophages, DC, fibroblasts and smooth muscle cells [12, 15-17]. To exert its chemotactic activity, CXCL16 is shed from the cell surface, a process that can be mediated by the protease ADAM-10 [18, 19]. In addition to its role as a chemokine ligand, CXCL16 (alternatively named SR-PSOX) acts as a scavenger receptor for oxidized LDL in its membrane-bound form [20] and might therefore play a role in the transformation of activated macrophages into cholesterol loaded foam cells. Interestingly, membrane-bound CXCL16 has been proposed to mediate adhesion to CXCR6+ cells [21], suggesting that it does not only attract T cells, but supports a prolonged interaction between APC and activated T cells as well.

Based on its potential to attract memory T cells, the CXCL16-CXCR6 pathway has been suggested to play an important role in the pathogenesis of several inflammatory diseases [12, 22-25]. We recently showed that CXCL16 is abundantly expressed in RA ST [12], which was confirmed by others [15, 25]. In addition, CXCL16 can be found in high amounts in the SF of RA patients [12, 15]. Interestingly, neutralization of CXCL16 recently proved to be beneficial in collagen induced arthritis, substantiating its potential as a therapeutic target [25]. Despite the large body of evidence for a role of CXCL16 in a variety of inflammatory diseases, little is known on the regulation of CXCL16 expression. However, cells originating from monocyte precursors are held responsible for a large part of the CXCL16 expression in the synovial cavity [12]. In this light, insight in CXCL16 regulation by these cells might help to explain the abundant expression

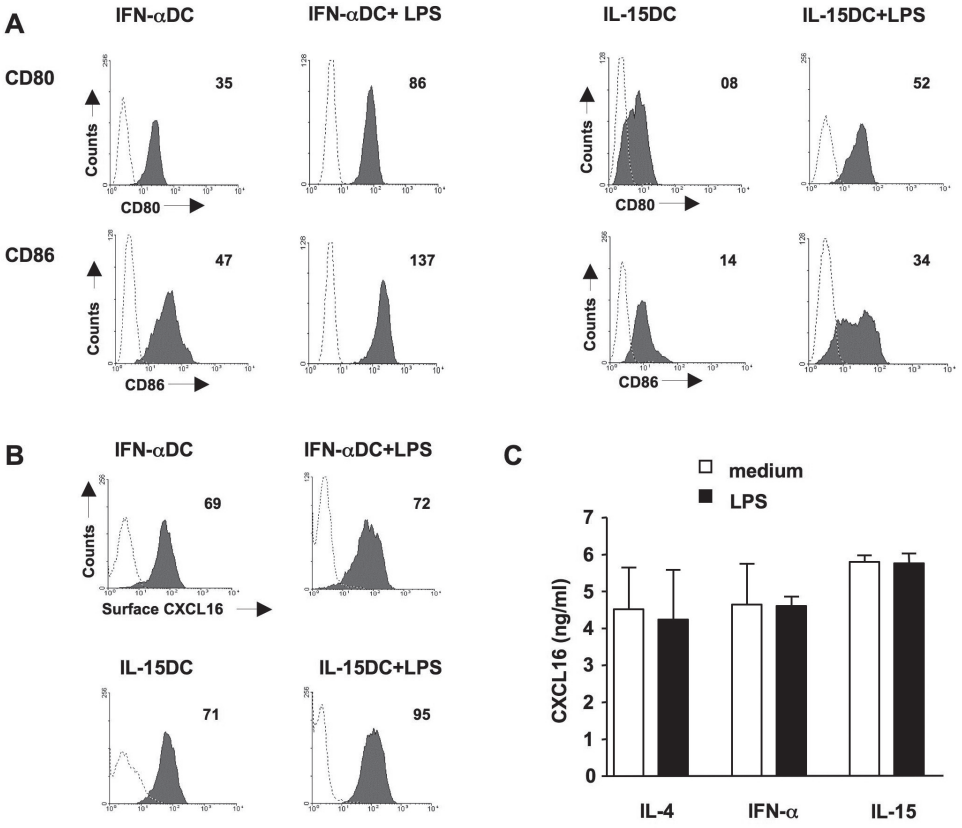

Figure 2:

CXCL16 expression and secretion by IFN- DC and IL-15DC

Panel A shows expression of CD80 and CD86 (shaded) vs. mouse IgG1 (dotted) by DC cultured with IL-15 (100 ng/ml) or IFN-alpha 500 U/ml) in combination with GM-CSF (500 U/ml) for 6 days and another 48 hours in the presence or absence of 2 µg/ml LPS. Representative data of 8 individual experiments are shown. Panel B shows representative CXCL16 membrane expression on day 8 with and without stimulation with 2 µg/ml of three individual experiments. Panel C shows mean soluble CXCL16 (±SEM) from day 6 to day 8 of 3 individual experiments. The accompanying Mean Fluorescence Intensity (MFI) is shown in de figure.

in this particular site. The aim of this study was therefore to elucidate the factors that regulate CXCL16 expression and release by monocytes and DC both in healthy individuals and in RA patients. Here, we provide novel data that contribute to the understanding of the complex regulation of CXCL16 in these cells. This regulation however is not altered in RA, suggesting that the increased CXCL16 expression in RA joints is predominantly caused by an increased influx of CXCL16 producing cells.

Methods

Patients and samples

For all blood cell cultures, 50 ml heparinized venous blood was taken from healthy volunteers and RA patients. SF was obtained from RA patients with an active local inflammation by means of puncture or during small needle arthroscopic procedures. All RA patients fulfilled the ACR criteria for RA 26 and for experiments with RA monocytes, only patients with an active disease (DAS-28 > 5.1) were included [27]. These patients were treated with NSAIDs and methotrexate and/or sulphasalazine and had not received treatment with biologicals. The study was approved by the local ethics committee. Informed consent was recorded from all patients who participated in the study.

Recombinant proteins and antibodies

For the generation of MoDC, the following recombinant human (Rh) proteins were used: GM-CSF (Strathmann Biotech, Hamburg Germany), IL-4 (Strathmann Biotec), IL-15 and IFN-αA2 (Ropheron A, Roche). For stimulating monocytes/macrophages, we used Rh IL-10, IL-13, IL-15, IL-18, TNF-α and IFN-γ (all R&D Systems, Abingdon, UK). For FACS analysis, goat anti-human CXCL16 (R&D Systems, Abingdon, UK) and mouse anti-human CD14 (Immunotech), CD80 (Becton Dickinson), CD86 (Pharmigen), CD83 (Beckman Coulter, Mijdrecht, The Netherlands), MHC-I (clone W6/32) and MHC-II (clone q1513) were used. As control antibodies for FACS, mIgG1, mIgG2a, mIgG2b, (all from BD Biosciences, Alphen ad Rijn, The Netherlands) and goat IgG (R&D Systems, Abingdon, UK) were used. Donkey anti-goat AlexaFluor 647 (Molecular Probes, Leiden, The Netherlands) and goat anti-mouse FITC (Zymed South San Francisco, CA, USA) were used as secondary antibodies. The goat anti-human CXCL16 antibody was also used as a detection antibody for ELISA. For the standard curve, RhCXCL16 (R&D Systems, Abingdon, UK) was used and biotinylated rabbit anti-human CXCL16 (Pepro Tech, Rocky Hill, USA) was used as a detection antibody.

Isolation of monocytes and generation of monocyte derived dendritic cells and monocytes/ macrophages

MoDC were cultured as described previously [28, 29]. In brief, mononuclear cells were isolated with a density gradient over Lymphoprep (Axis-Shield, Oslo, Norway). Mononuclear cells were isolated by adherence and were cultured in medium enriched with 10% FCS (Greiner), 500 U/ml GM-CSF and 350 U/ml IL-4, 100 ng/ml IL-15 or 500 U/ml IFN-α. For maturation, cells were cultured for another 2 days in the presence of cytokines and 2 μg/ml LPS. For experiments with monocytes/macrophages, CD14+ cells were isolated by means of magnetic cell separation (MACS) (Miltenyi Biotec). Cells were stimulated with 350 U/ml IL-4, 20 ng/ml IL-10, 20 ng/ml IL-13, 20 ng/ml IL-15, 20 ng/ml IL-18, 20 ng/ml TNF-α or 10 ng/ml IFN-γ as indicated.

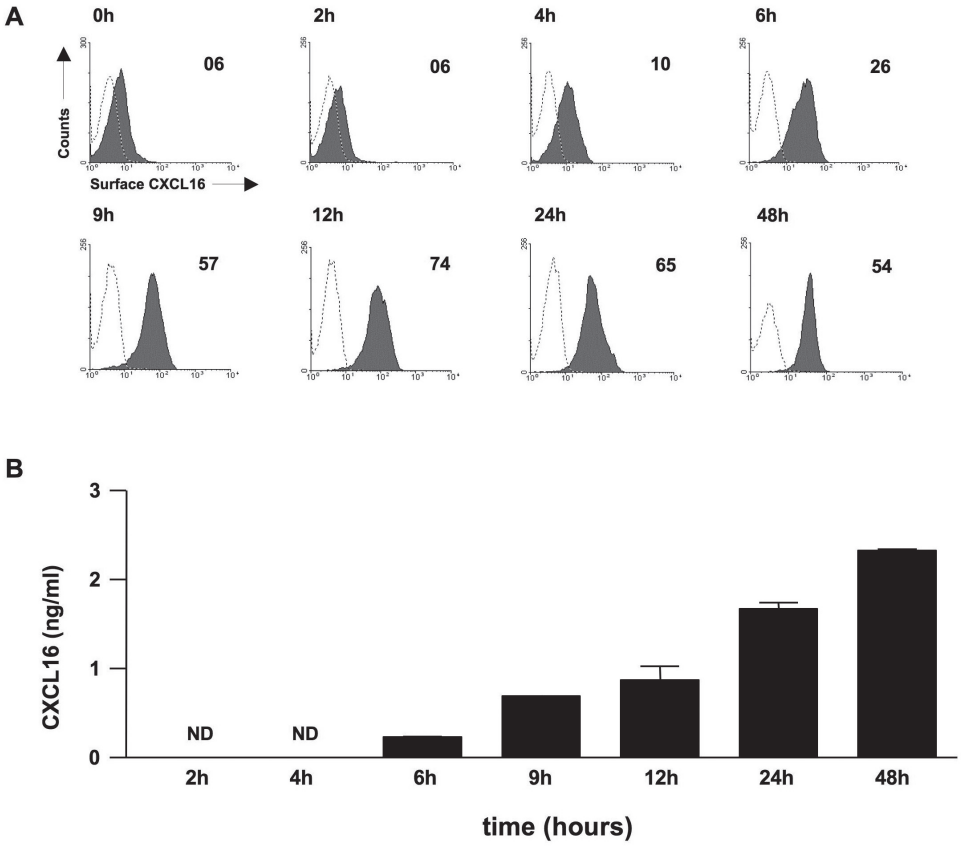

Figure 3:

CXCL16 expression and secretion by monocytes/macrophages

Panel A shows increasing CXCL16 expression (shaded) vs. goat IgG (dotted) on freshly isolated monocytes that were cultured for 48 hours. The accompanying Mean Fluorescence Intensity (MFI) is shown in de figure. Panel B shows cumulative CXCL16 release over the first 48 hours. The figure is representative for 5 individual experiments.

Synovial Fluid cell isolation

Synovial fluid samples were centrifuged and cells were extensively washed in PBS. Synovial fluid mononuclear cells (SFMC) from RA SF were obtained by density gradient centrifugation over Lymphoprep. SFMC were washed with PBS, and either used immediately for FACS analyses or prepared for CD14+ MACS isolation. SFMC and CD14+ SF cells were either directly prepared for FACS analysis or cultured for 24 hours where indicated.

Fluorescent Activated Cell Sorting (FACS)
For FACS analysis, cells were prepared as described previously [28]. In brief, cells were incubated with primary antibody or matched isotype control (normal goat IgG for CXCL16) for 30 minutes on melting ice. Cells were washed and incubated with a secondary antibody for another 30 minutes. After a final wash step, cells were transferred into FACS tubes and analyzed on a FACS Calibur (Becton Dickinson).

Enzyme Linked Immuno Sorbent Assay (ELISA)
CXCL16 detection in cell supernatant was performed with a sandwich ELISA as described previously [30]. Samples were measured in duplicate or triplicate and pooled normal human serum was taken along as an internal control on every plate. The detection limit of our ELISA is 100 pg/ml.

Statistical Analysis
Differences between soluble CXCL16 secretion levels between different stimulations were tested for significance with a Wilcoxon's signed rank test or a paired t test when appropriate (figure 2c). Differences in CXCL16 surface expression between RA monocytes and SF CD14+ cells were tested for statistical significance using a Mann Whitney U test. P-values <0.05 were considered significant.

Results

CXCL16 is expressed and secreted by monocyte derived dendritic cells
To study CXCL16 expression by MoDC, adherent monocytes from 10 healthy individuals were cultured in the presence of IL-4 and GM-CSF for 6 days, resulting in MoDC with an immature phenotype (data not shown). Upon additional stimulation with LPS for 2 days, immature DC developed a mature phenotype with up-regulation of co-stimulatory molecules (CD80 and CD86), the mature DC marker CD83 and MHC molecules (data not shown). Interestingly, we found that CXCL16 surface expression was comparable for mature and immature MoDC (n=10, figure 1a). In line with our FACS results, CXCL16 secretion by DC matured with LPS was not different from CXCL16 release by immature DC (n=6, figure 1b), which is in contrast to the increased secretion observed for many other chemokines and cytokines [9, 28, 31].

In RA, monocytes encounter a large variety of cytokines, potentially skewing their differentiation towards a DC phenotype. In addition, culturing with IL-4 may introduce an in vitro bias with regard to CXCL16 expression by MoDC. Therefore we examined CXCL16 expression and secretion by MoDC cultured from 3 healthy individuals in the presence of IFN-α or IL-15 [32, 33], both of which have been suggested to play a role in the pathogenesis of RA [34, 35]. We will refer to these cells as IFN-αDC

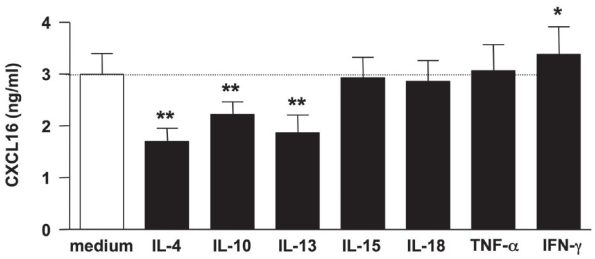

Figure 4:

Th2 cytokines inhibit CXCL16 secretion by monocytes/macrophages

The figure depicts mean (±SEM) (n=8) secreted CXCL16 levels (ng/ml) of monocytes that were cultured for 36 hours in medium and stimulated with IL-4 (300 U/ml), IL-10 (20 ng/ml), IL-13 (20ng/ml), IL-15 (20 ng/ml), IL-18 (20ng/ml), TNF-alpha (20ng/ml) or IFN-gamma (10 ng/ml). *= $p<0.05$, **= $P<0.01$.

and IL-15DC throughout the manuscript. Both these DC types express low levels of the co-stimulatory molecules CD80 and CD86. Upon stimulation with LPS, DC exhibit a clearly increased surface expression of CD80 and CD86 (figure 2a). With regard to CXCL16 expression, we did not observe significant differences between IL-15DC, IFN-αDC and DC cultured with IL-4 (figure 2b), although IL-15DC exhibited somewhat higher CXCL16 expression in an occasional donor. Similarly, CXCL16 secretion by IL-15DC was somewhat higher than CXCL16 secretion by IL-4DC or IFN-αDC, but this difference was not statistically significant (n=3, figure 2c). In line with MoDC cultured with IL-4, stimulating IFN-αDC and IL-15DC with LPS for 2 days did not result in consistent effects on CXCL16 expression or secretion (figure 2b,c), indicating that CXCL16 expression and secretion is similar on immature and mature MoDC generated at different conditions.

Rapid increase of CXCL16 expression and secretion by cultured monocytes

Since CXCL16 expression was comparable on distinct MoDC, we next investigated how trans-membrane and soluble CXCL16 is regulated on their predecessors. Freshly isolated monocytes weakly expressed CXCL16 on their cell surface. Interestingly, a clear up-regulation of CXCL16 surface expression could already be observed within 4 hours of culture in medium without cytokines, resulting in a full blown expression in as little as 12 hours. This expression was consistent over time and the level of expression resembled CXCL16 expression on MoDC (n=5, figure 3a). This upregulation of CXCL16 expression was closely followed by CXCL16 protein release, as we could detect significant amounts of CXCL16 after as little as 4-6 hours, with a clear cumulative effect over time (figure 3b). In line with our previous findings [12], the protease ADAM-10 could not be detected on the surface of monocytes/macrophages

using flowcytometry within the first 48 hours of culture (data not shown), suggesting that other pathways of CXCL16 shedding must be involved. The rapid increase in CXCL16 expression and secretion was not due to culture effects, as adherent monocytes, monocytes isolated with negative selection or exchanging FCS for human serum had a similar CXCL16 expression pattern and kinetics (data not shown). In order to examine the possibility that CXCL16 protein is already stored intracellularly by monocytes, we performed an intracellular FACS staining on fresh PBMC's that were gated on CD14 expression. Interestingly, the intracellular expression was similar to membrane expression (data not shown), suggesting that CXCL16 protein is not stored in large amounts to be transported to the membrane but is actually produced *de novo* upon culture of monocytes.

Inhibition of CXCL16 secretion by Th2/anti-inflammatory cytokines and stimulation by the Th1 cytokine IFN-γ

To date, little is known on the effects of cytokines on CXCL16 expression and release by human monocytes/macrophages. Moreover, no data are available on the first 48 hours, where the most profound up-regulation takes place (figure 3). Therefore we investigated the effect of pro- and anti-inflammatory cytokines on trans-membrane and soluble CXCL16 levels. In the supernatant of 36 hour monocytes/macrophages (n=8), we measured a CXCL16 release of 3.0 ±0.4 ng/ml (mean ±SEM). Interestingly, the Th2 cytokines IL-4 and IL-13 inhibited CXCL16 release with 43% (1.7 ±0.3 ng/ml, p=0.008) and 38% (1.9 ±0.3 ng/ml, p=0.008) respectively (figure 4). Furthermore, the anti-inflammatory/regulatory cytokine IL-10 inhibited soluble CXCL16 release on developing monocytes/macrophages with 26% (2.2 ±0.2 ng/ml, p=0.008). In contrast, stimulation with the Th1 cytokine IFN-γ resulted in an increase of 13% (3.4 ±0.5 ng/ml, p=0.02) compared with medium alone (figure 4). This effect could not be observed for the pro-inflammatory cytokines TNF-α, IL-15 and IL-18, indicating that soluble CXCL16 release is uniquely upregulated by IFN-γ. Using flowcytometry, a maximal CXCL16 membrane expression could be observed on monocytes/macrophages at 36 hours, irrespective of stimulation with pro- or anti-inflammatory cytokines (data not shown).

LPS inhibits CXCL16 expression and secretion by monocytes

To further investigate the effects of monocyte activation on CXCL16 expression, we incubated freshly isolated monocytes with the TLR4 ligand LPS. Intriguingly, LPS clearly delayed the upregulation of CXCL16 expression within the first 48 hours (n=3, figure 5a). The inhibition of surface expression however was transient, as the expression after 48 hours was comparable to the expression on unstimulated monocytes/macrophages. When measured at 36 hours in 12 healthy individuals, LPS resulted in a significant inhibition of (mean ±SEM) CXCL16 release (1.9 ±0.3 ng/ml)

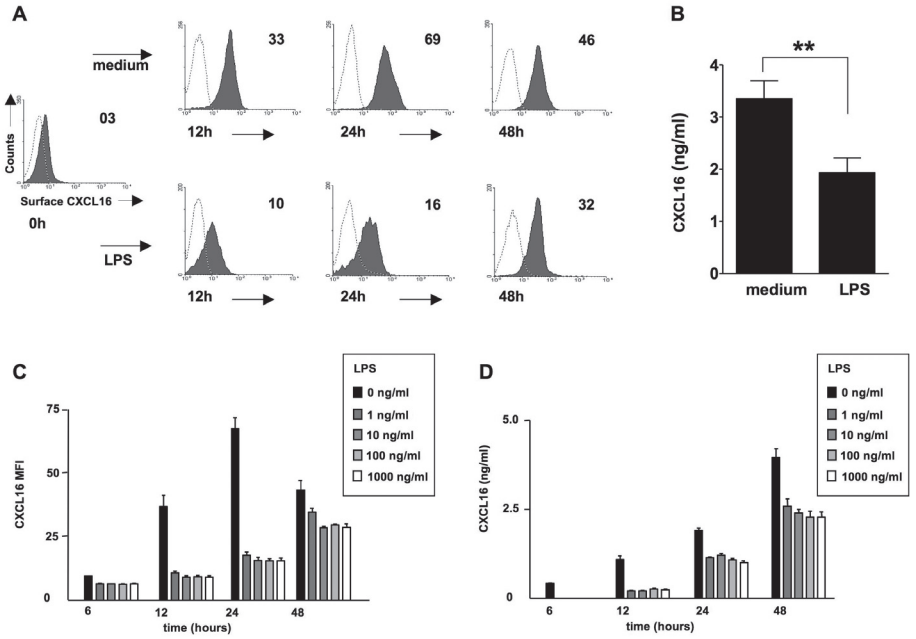

Figure 5:

LPS inhibits CXCL16 expression and secretion

Panel A depicts CXCL16 surface expression (shaded) vs. goat IgG (dotted) of monocytes/macrophages that were culture for 48 hours in the presence (bottom) or absence (top) of LPS (100 ng/ml). Representative result of 3 individual experiments is shown. Panel B shows CXCL16 secretion (ng/ml) by monocytes/macrophages cultured for 36 hours in the presence or absence of 100 ng/ml LPS (n=12)) **p<0.01. Panel C depicts mean fluorescence intensity (MFI) of monocytes/macrophages cultured for 48 hours in the presence of LPS in increasing concentrations (0-1000 ng/ml). Mean (±SEM) values of 3 individual experiments are shown. Panel D depicts soluble CXCL16 (ng/ml) released by monocytes/macrophages cultured for 48 hours in the presence of LPS in increasing concentrations (0-1000 ng/ml). Mean (±SEM) values of 3 individual experiments are shown. The accompanying Mean Fluorescence Intensity (MFI) is shown in de figure.

compared to unstimulated monoctyes (3.4 ±0.4 ng/ml) (p=0.001) (figure 5b). In a concentration/time-course experiment, stimulation with 1 ng/ml LPS resulted in the same effects as stimulation with 1000 ng/ml on both CXCL16 membrane expression (n=3, figure 5c) and secretion (n=3, figure 5d), indicating that an initial stimulation with a relatively low concentration of LPS is sufficient to delay trans-membrane expression and soluble CXCL16 release by cultured monocytes.

CXCL16 expression and secretion is not altered in RA

In order to explain the increased CXCL16 expression in RA [12], we tested whether CXCL16 was differentially regulated on blood monocytes from RA patients compa-

red to healthy controls. Freshly isolated monocytes from 3 RA patients with active disease showed a similar low surface expression of CXCL16 as monocytes from 3 healthy controls (figure 6a). Furthermore, CXCL16 expression followed similar kinetics in RA monocyes as in monocytes from healthy controls (figure 6a). Interestingly, CD14+ cells isolated from RA synovial fluid (n=5) had a CXCL16 expression level (median (range)) that was slightly but significantly higher than the expression level on peripheral blood monocytes (5.0 (4.5-7.0) vs. 10.0 (8.0-15.0) (p=0.03)) (figure 6a and 6b), but clearly lower than cultured macrophages beyond 12 hours. Upon culture for 24 hours, SF CD14+ cells further upregulated CXCL16 surface expression in a similar way as peripheral blood monocytes (figure 6b). In contrast, SF lymphocytes did not express CXCL16 (data not shown), which is in line with peripheral blood lymphocytes [12]. As stimulation with LPS resulted in the clearest effects on CXCL16 expression and secretion, we stimulated RA monocytes with LPS. Comparable to monocytes from healthy controls, the normal increase in CXCL16 expression and release by RA monocytes (n=3) was delayed by LPS (figure 6c&d).

Discussion

CXCL16 is expressed by myeloid cells and is abundant in RA joints [12, 15, 25]. In the present study, we provide novel data that contribute to the understanding of CXCL16 regulation in RA patients and healthy controls. First, we show that CXCL16 expression and release is conserved on distinct myeloid DC and not affected by maturation. Second, we demonstrate that monocytes rapidly upregulate CXCL16 expression and release when brought into culture, which is transiently abrogated by LPS. Third, we show that Th2 cytokines suppress CXCL16 secretion while the Th1 cytokine IFN-γ enhances CXCL16 secretion by monocytes/macrophages. Finally, we demonstrate that RA monocytes express similar levels of CXCL16 compared to monocytes from healthy controls and that CXCL16 regulation is not altered in RA monocytes compared to monocytes of healthy controls.

Monocytes differentiate into macrophages or DC upon leaving the bloodstream and entering the tissues. Culturing monocytes/macrophages *in vitro* is considered to mimic this monocyte to macrophage development *in vivo* [36, 37]. In our experiments, this model for monocyte activation was the strongest trigger for upregulation of CXCL16 surface expression. Maximal CXCL16 surface expression was observed after as little as 12 hours and expression was highly stable and conserved on distinct monocyte derived cells, e.g. independent of DC or macrophage differentiation. This suggests that differentiation of monocytes, which also occurs by entering the tissues, is the main trigger for upregulation of CXCL16 expression, which may explain the abundant CXCL16 expression in various tissues [24, 38], both in health and disease. The exact mechanism of

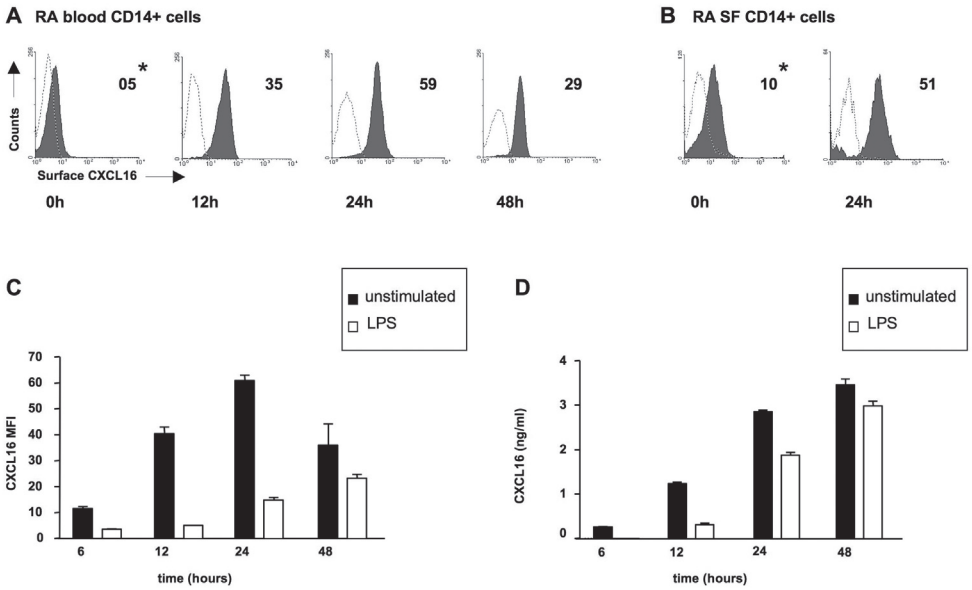

Figure 6:

CXCL16 regulation is not altered in RA

Panel A depicts CXCL16 expression (shaded) vs. goat IgG (dotted) on monocytes/ macrophages from RA patients that were cultured for 48 hours. Representative figure of 3 individual experiments is shown. Panel B shows CXCL16 expression on freshly isolated SF CD14+ cells (left) and SF CD14+ cells cultured for 24 hours (right). Representative figure of 5 experiments is shown. *Difference between blood CD14+ cells (figure 6A, first plot) and SF CD14+ cells (figure 6B, first plot) is significant (p=0.03). Panel C depicts CXCL16 mean fluorescence intensity (MFI) of RA monocytes/macrophages that were cultured in the presence of 100 ng/ml LPS (0-1000 ng/ml) for 48 hours. Mean (±SEM) values of 3 individual experiments are shown. Panel D depicts soluble CXCL16 (ng/ml) released by RA monocytes/macrophages that were cultured in the presence of 100 ng/ml LPS (0-1000 ng/ml) for 48 hours. Mean (±SEM) values of 3 individual experiments are shown

this increase remains to be elucidated, but our results suggest that rapid de novo production rather than stored intracellular CXCL16 protein is responsible for the increase of CXCL16 expression upon culturing monocytes. Interestingly, CD14+ cells isolated from RA SF had a CXCL16 expression that was slightly higher than blood monocytes but lower than cultured macrophages, which might be explained by a certain state of activation of CD14+ cells in RA SF. In our experiments, we detected large amounts of soluble CXCL16, while we could not detect ADAM-10 expression within the first 48 hours of culture. This may indicate that other pathways, for example alternative splicing or other proteases, contribute to the release of the extracellular CXCL16 domain.

To date, little is known about CXCL16 regulation on myeloid cells. In our study, we observed a small but significant increase in CXCL16 secretion upon stimulation

with IFN-γ, suggesting that this Th1 cytokine does have some stimulatory effect on CXCL16 levels. However, this effect on monocytes is only small compared to the effect of merely culturing monocytes. Interestingly, we observed an inhibition of CXCL16 release by the Th2 cytokines IL-4 and IL-13 and the anti-inflammatory cytokine IL-10. This indicates that Th1 vs. Th2 polarisation can enhance or inhibit soluble CXCL16 to some extend, which may affect local CXCL16 levels. Stimulation with TNF-α, IL-15, IL-18 or IFN-α, which all have been suggested to play a significant role in RA [34, 35, 39-43], had little or no effect on CXCL16 expression or secretion. The lack of CXCL16 inducing effect of TNF-α seems in contrast with our previous findings. However, this apparent inconsistence can be explained by temporal factors, as CXCL16 expression did not increase upon stimulation with TNF-α, but decreased without additional cytokine stimulation after 48 hours, with a both a CXCL16 positive and negative fraction [12]. This CXCL16 negative fraction of unstimulated monocytes lost viability beyond 48-72 hours, which could be prevented by stimulation with TNF-α but also growth factors such as (G)M-CSF (data not shown). Thus TNF-α does not exert a direct CXCL16 inducing effect on cultured monocytes/macrophages.

While the Th2/anti inflammatory cytokines IL-4/IL-13/IL-10 inhibited soluble CXCL16 secretion by monocytes to some extend, the TLR4 ligand LPS clearly delayed CXCL16 expression and secretion. Considering the data from the present study, it cannot be excluded that this effect is indirectly caused by cytokines such as IL-10. Alternatively, one can speculate that LPS, as a strong initiator of innate immunity, delays upregulation of CXCL16 expression and secretion to allow optimal orchestration of an innate immune response. On DC, we did not detect significant effects on CXCL16 upon maturation with LPS. This is likely to be explained by a ceiling effect, as CXCL16 expression already is maximal on these cells. In addition, we did not observe significant differences between mature and immature DC on soluble CXCL16 levels, indicating that DC maturation does not influence CXCL16 expression or secretion in any way.

In previous studies, it was shown that circulating CXCL16 serum levels are not altered in patients with active RA compared to healthy controls [15, 30]. In contrast, local expression in the ST is increased [12], as is the expression of the receptor CXCR6 in the SF [12]. The data of the present study provide no evidence for deregulation of CXCL16 expression in RA monocytes. Given these data, it is conceivable that increased CXCL16 expression in RA joints is caused by influx of monocytes rather than enhanced production per cell. The observation that RA patients responding to anti-TNF-α treatment did have a decreased CXCL16 expression in the ST compared to non-responders [12] may be caused by a difference in monocyte numbers, either by decreases in influx or enhanced apoptosis. Further studies using synovial biopsies

during DMARD or anti-TNF-α treatment need to be performed to confirm this relationship and to value the potency of targeting CXCL16 or its receptor CXCR6 as a future therapy for RA.

In summary, we provide evidence suggesting that monocytes rapidly upregulate CXCL16 upon differentiation into macrophage or DC, which can be transiently inhibited by mimicking innate immune activation using LPS. Soluble CXCL16 release is inhibited by Th2/anti inflammatory cytokines and stimulated by the Th1 cytokine IFN-γ to some extend. Together our data suggest that the abundant expression of CXCL16 in RA joints might be the result of abundance of infiltrating monocytes/macrophages in the synovial tissue, as no evidence for deregulation of CXCL16 in RA was observed.

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chapter 6

CIRCULATING LEVELS OF THE CHEMOKINE CCL18, BUT NOT CXCL16 ARE ELEVATED AND CORRELATE WITH DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS

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Abstract

Background: Antigen presenting cells (APC) and T cells are considered to play a significant role in the pathogenesis of rheumatoid arthritis (RA). CCL18 and CXCL16 are two chemokines that facilitate T cell attraction by APC, of which a role in the pathogenesis of RA has been suggested.

Objective: To compare the circulating levels of CXCL16 and CCL18 in RA with controls and to investigate the relation of CXCL16 and CCL18 with RA disease activity and joint damage.

Methods: Circulating CCL18 and CXCL16 levels were determined in 61 RA patients with a follow-up of 6 years and a group of 41 healthy controls with ELISA. Chemokine levels were correlated with demographical data, disease activity (DAS28) and joint damage (modified Sharp score). In addition, serum CCL18 and CXCL16 levels from a cohort of 44 RA patients treated with anti TNF-α were correlated with disease activity.

Results: CCL18 levels in serum were significantly elevated in RA patients compared to controls, while serum CXCL16 levels were not. In contrast to CXCL16, serum CCL18 was positively correlated with disease activity. Both CCL18 and CXCL16 levels decreased upon treatment with anti TNF-α. Neither CCL18 nor CXCL16 correlated with joint damage and progression.

Conclusion: Here, we show, for the first time, that circulating CCL18 and not CXCL16 levels are elevated in RA patients as compared with controls and correlate with disease activity in RA. More knowledge regarding the regulation and function of both CCL18 and CXCL16 is essential to value their role in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic auto-immune disease, characterized by an inflammation of the synovial joints that eventually leads to cartilage damage and bone destruction. Despite extensive research, the exact pathogenesis of RA is still unclear. Nowadays, there is substantial evidence supporting a role for antigen presenting cells (APC), such as dendritic cells (DC) and macrophages (MΦ) in RA [1-3]. These APC activate T cells and subsequently play a pivotal role in orchestrating immune responses [4]. In addition, upon stimulation by T cells, APC act as downstream players in RA and secrete cytokines such as TNF-α and IL-1β, which are now successfully targeted in the clinic [5, 6]. In order to direct T cell responses, APC first need to attract different T cell subsets. This chemo-attraction is mediated by chemokines (CK). CK constitute a large family of proteins that all possess chemo-attractive capacities towards leucocytes. A subset of the CK family preferentially attracts T cells and is therefore critical in the direction of T cell-driven immune responses.

Recently, we started investigating the role of DC and a large panel of T cell attracting chemokines secreted by those DC in RA, of which CXC chemokine ligand 16 (CXCL16) and CC Chemokine ligand 18 (CCL18) were identified as particularly interesting subjects [7-11]. CXCL16 is a unique trans-membrane CK exists in both a membrane bound and soluble form. Membrane bound CXCL16 is a scavenger receptor for oxidized low density lipoproteins (LDL) [12], can facilitate cell adhesion [13] and mediates phagocytosis of bacterial fragments [14]. This membrane-bound CXCL16 is expressed on APC 9, 15, 16 and can be cleaved by proteases such as ADAM-10 [17, 18] to serve as a chemo-attractant for CXCR6+ cells in its soluble form [16, 19]. The receptor CXCR6 is present on activated, memory type T cells, plasma cells and NKT cells [19-21]. We recently demonstrated the abundant expression of CXCL16 and CXCR6 in RA synovial tissue and fluid, as well as its regulation by synovial fluid and TNF-α [9]. In addition, we found high levels of cleaved CXCL16 in the synovial fluid of RA patients. Recently, additional evidence for a role for CXCL16 in RA was provided as CXCL16 was suggested as a potentially novel therapeutic target in RA as blockade of CXCL16 resulted in a decrease in arthritis in murine collagen induced arthritis [22].

CCL18 (DC-CK1, PARC, AMAC-1) is a another T cell attracting CK that was first identified as a chemo attractant for naïve T cells and is produced by DC and alternatively activated MΦ [23-26]. CCL18 was initially found in high quantities in the lung (alveolar MΦ) in health and disease [24]. Interestingly, a high CCL18 expression was found in synovial tissue of patients with RA [7, 27], which suggested that CCL18 might play a role in the pathogenesis of RA. In addition, CCL18 can also act as a pro-fibrotic factor in the lung [28], indicating that T cell attraction is not the only function of CCL18. Circulating CCL18 has been shown to be useful as a biomarker for Gaucher's

disease [29]. Moreover, associations with circulating CCL18 levels have been suggested in a large variety of diseases, including lymphoblastic leukemia [30], atopic dermatitis [31], ovarian carcinomas [32] and allergic asthma [33].

Sensitive biomarkers for disease activity and progression in RA are currently still lacking. The demand for such markers, however, is increasing, since novel therapeutic strategies are very expensive, have serious side effects and vary in efficacy between individual patients. This, in combination with the suggested roles of CXCL16 and CCL18 in RA pathogenesis prompted us to investigate their circulating levels in RA and their potential correlation with clinical disease parameters. In the present study, we show that serum levels CCL18 are elevated in RA and significantly correlate with disease activity parameters in two independent cohorts, whereas CXCL16 did not show any correlation with disease activity. Neither of the two CK correlated with radiological progression. These results suggest that serum CCL18 but not CXCL16 might reflect the disease course of RA.

Methods

Patients

Patient serum samples were repetitively taken from 61 patients enrolled in the RA inception cohort of the Radboud University Nijmegen Medical Centre. All patients fulfilled the ACR criteria for the diagnosis RA [34]. The first sample was taken at the time of diagnosis, prior to the initiation of treatment with disease modifying anti rheumatic drugs (DMARDs), and is referred to as baseline sample throughout the manuscript. None of the patients had been treated with anti TNF-α during the period of follow-up we analyzed in our study. Patients were seen on a regular basis and data were collected every 3 months during the first two years and every 6 months thereafter. In this cohort, serum samples can be correlated with clinical data from the same day of the blood sample, such as disease activity (DAS-28 score [35]), joint damage (modified Sharp score [36]) and laboratory values. Serum samples of healthy volunteers (n=41) were used as controls to compare CK levels with RA serum levels. In addition, serum samples were taken from 44 patients who where treated with anti-TNF-α (infliximab) in the St. Maartenshospital. The first sample was taken before the first infusion and the next samples were taken after 2, 6 and 14 weeks. The ESR was measured on the same time points, as were the joint scores and VAS for the DAS-28 score.

Enzyme Linked Immuno Sorbent Assay (ELISA)

For the detection of chemokine protein levels of CXCL16 and CCL18 in serum, sandwich ELISA's were performed as described previously [9, 37]. As an internal control for inter-assay variability, a sample of pooled normal human serum (n=300) was taken

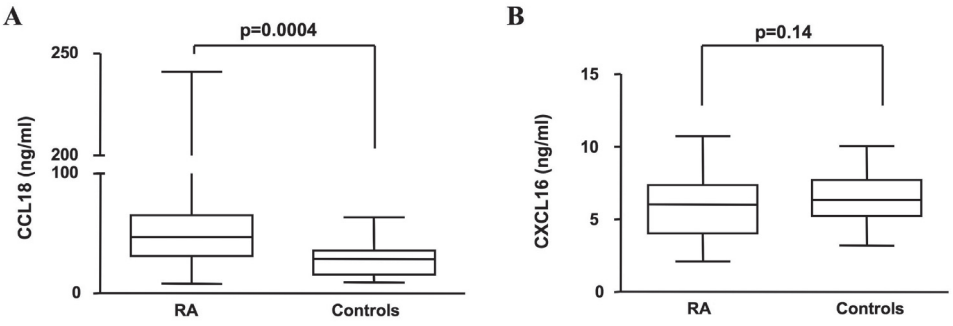

Figure 1:

Serum chemokine levels in RA vs. controls

Serum chemokine levels were measured in RA patients prior to DMARD treatment and in healthy controls. Panel A depicts CCL18 levels and panel B depicts CXCL16 levels in serum from RA patients (n=61) and controls (n=41). Box & whiskers plots show median levels with interquartile range and full range. Levels were compared with a Mann Whitney U test Exact p-values are provided in the figure.

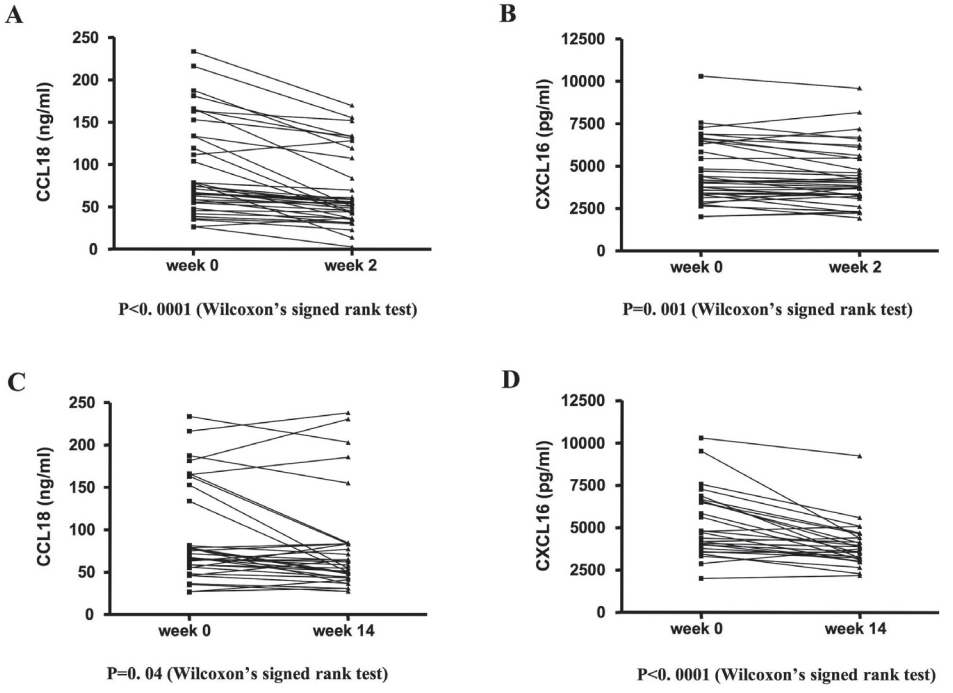

Figure 2:

Serum chemokine levels during anti TNF-alpha treatment

Chemokine levels were measured before and during treatment with infliximab. Panel A & B depict serum CCL18 and CXCL16 at baseline vs. week 2 and panels C & D depict serum CCL18 and CXCL16 levels at baseline vs. week 14. Levels were compared with a Wilcoxon's signed rank test. Exact p-values are provided in the figure.

along in all assays. The detection limits for the ELISA's is 100 pg/ml for both CCL18 and CXCL16. The maximum inter-assay variability is estimated at 10%. In order to minimize effects of this variability, samples that were directly compared were measured in the same assay.

Statistical analysis

In order to evaluate whether circulating chemokine levels were different in RA patients prior to DMARD treatment compared to healthy controls, baseline serum samples from patients were compared with control serum samples with a Mann Whitney U test. When examining the relation between circulating chemokine levels with disease activity and joint damage, correlations between chemokine levels and clinical data were determined with Pearson's correlation. CCL18 and modified Sharp scores were root transformed. To asses differences between chemokine levels at different time-points in our cohort of patients treated with anti-TNF-α, comparison between baseline and follow-up data was done with a Wilcoxon's signed rank test. The correlation between changes in DAS-28 and changes in CCL18 or CXCL16 levels was done with a Spearman's correlation test.

Results

Circulating CCL18 but not CXCL16 levels are elevated in RA

CCL18 and CXCL16 levels prior to DMARD treatment were measured in serum of 61 patients and compared with serum of healthy controls (n=41). At baseline, CCL18 levels were significantly higher in RA patients (median (interquartile range) 49.0 ng/ml (31.5-71.0)) compared to CCL18 levels in controls (28.6 ng/ml (15.8-35.7)) (p=0.0004) (figure 1a). In contrast, CXCL16 levels were not significantly elevated in RA patients (6.0 (4.0-7.3) ng/ml) compared to healthy controls (6.3 (5.2-7.7) ng/ml) (p=0.14) (figure 1b). Neither CCL18 nor CXCL16 levels were associated with demographic or patient characteristics, including rheumatoid factor positivity, age at disease onset and gender as calculated by univariate analysis (data not shown).

CCL18 but not CXCL16 correlates with the disease activity score (DAS-28)

Since we were interested in the relation with disease severity and potential suitability of CCL18 and/or CXCL16 as a biomarker for disease severity in RA, we investigated whether circulating levels of these CK correlated with clinical parameters at baseline and over 3 and 6 years. In table 1, patient and disease characteristics are shown at baseline and over time for progression of joint damage.

SqrtCCL18 at baseline was positively correlated with the DAS-28 score (R=+0.38 (p =0.003)) and ESR (+0.39 (p =0.003)) (upper panel of table 2). In contrast, baseline serum CXCL16 levels did not correlate with disease activity. As could be expected

Table 1. Demographical data and disease phenotype

Age (yrs)	51	(14)
Gender (female)	38	(62%)
RF positive (yes)	42	(69%)
ESR (mm/h)	38	(20-58)
DAS28	5.5	(1.4)
Sharp 0	12	(4-20)
ΔSharp 0-3	35	(7-88)
ΔSharp 0-6	40	(19-87)

N=61 Values are numbers (%), mean (SD) or median (interquartile range)

Table 2. Correlations of baseline chemokine levels with disease activity, joint damage and progression of joint damage

	CXCL16	CCL18
ESR	-0.03	**0.39**
DAS28	-0.05	**0.38**
Sharp 0	0.03	0.06
ΔSharp 0-3	0.00	0.14
ΔSharp 0-6	0.06	0.10

Pearson's correlations, with significant ($p<0.05$) correlations printed bold.
CCL18 and Sharp scores were root-transformed.

considering their regulation, CCL18 and CXCL16 were inversely correlated but this correlation did not reach statistical significance (R=-0.22 (p=0.10)).

Over time, a trend towards a positive correlation between mean serum CCL18 and mean DAS-28 could be observed, although its was less strong compared to baseline (R=+0.21 (p= 0.10) over 3 years (R=+0.23 (p =0.07) over 6 years) (upper panel of table 3). As for baseline, mean CXCL16 over time did not correlate with mean DAS-28 (R=+0.06 (p=0.63) and +0.02 (p= 0.86) over 3 and 6 years respectively). Mean

CXCL16 and mean CCL18 again showed a trend towards an inverse correlation (R=-0.21 (p =0.10) and -0. 20 (p=0.12) over 3 and 6 years, respectively).

Serum chemokine levels do not correlate with joint damage and progression
Cartilage and bone damage are important clinical outcomes in the chronic process of RA. In order to investigate whether serum CK levels could predict joint damage, we correlated CK levels at baseline and during the follow-up period with modified Sharp-scores and progression. In the literature, female gender and rheumatoid factor are known for their positive relation with progression of joint damage in RA [36] and might therefore act as confounding factors. However, the levels of CCL18 and CXCL16 were not related to gender or rheumatoid factor in our study population (data not shown). In these patients, neither CCL18 nor CXCL16 correlated significantly with Sharp score at baseline (lower panel of table 2). For comparison, the correlation of baseline DAS28 with progression in Sharp score between 0-3 years and 0-6 years was R=0.36 (p=0.005) and R=0.39 (p=0.002), respectively. Also, the CK levels over time, averaged over 3 years and 6 years, did not correlate with progression of joint damage over the same time period, in contrast to the DAS28 (lower panel of table 3).

CCL18 decreases upon anti TNF-α treatment and correlates with DAS-28
Anti-TNF-α treatment is known for its strong and rapid effects on disease activity in RA. Furthermore, TNF-α has been suggested to play a role in the regulation of expression of both CCL18 [10] and CXCL16 [9, 17]. To investigate the relation between chemokine levels and disease activity during treatment with anti TNF-α, we measured these parameters in a group of RA patients that were treated with infliximab (n=44). Interestingly, in 95% of the patients (35/37, for 7 patients week 2 serum was not available), CCL18 levels dropped significantly (p<0.0001) after the initiation of anti TNF-α treatment (figure 2a). In this period, the change in CCL18 was positively correlated with the change in DAS-28 (R=+0.38 (p=0.04)). CXCL16 levels were also significantly lower (p=0.04) at week 2, but the difference was small (median 4118 pg/ml vs. 3928 pg/ml). Moreover, CXCL16 dropped in only 65% (24/37) patients after initiation of treatment and increased in 13 patients (figure 2b) and a correlation with DAS-28 did not reach statistical significance (R=+0.38 (p=0.07)). The CCL18 changes over the first two weeks of treatment did not evolve in a common pattern that could be observed throughout the whole follow-up. After 14 weeks of treatment, CCL18 and CXCL16 levels were still significantly lower than baseline CCL18 levels (figure 2c&d). However, the change in CCL18 after 14 weeks did not correlate significantly with a change in DAS-28 (R=0.15 (p=0.5)). As for CCL18, CXCL16 levels at week 14 did also not correlate significantly with DAS-28 (R= -0.03 p=0.8).

Table 3. Patient averaged levels of chemokines and DAS28 over time correlated with progression of joint damage

	Time (years)	mCXCL16	mCCL18	mDAS-28
mDAS28	0-3	0.06	**0.21**	-/-
mDAS28	0-6	0.02	**0.23**	-/-
ΔSharp	0-3	0.11	0.14	**0.59**
ΔSharp	0-6	0.14	0.14	**0.52**

Pearson's correlations, with significant ($p<0.05$) correlations printed bold. CCL18 and Sharp scores were root-transformed.

Discussion

In the present study, we show that circulating CCL18 but not CXCL16 levels are elevated in RA patients compared with controls and correlate with disease activity. In addition, neither CXCL16 nor CCL18 correlated with the level of joint damage or, more importantly, the progression of such damage.

The correlation between CCL18 and disease activity might reflect a role for CCL18 in the pathogenesis of RA. The interpretation of this observation however is difficult and hampered by the lack of knowledge on the exact role of CCL18 in the immune system, which might be either pro- or anti- inflammatory. It is tempting to speculate that CCL18 acts as an anti inflammatory mediator in RA since its production is regulated by IL-4, IL-13 and IL-10 [11, 25], which places CCL18 in a Th2 or regulatory corner. This would imply that serum CCL18 levels may lag behind of disease activity in time. However, these thoughts are hypothetical and have not been proved so far in experimental settings. On the other hand, CCL18 might also elicit a pro-inflammatory response in RA, since the inflammatory environment might direct newly attracted T cells by CCL18 into an undesirable state of activation, subsequently resulting in ongoing T cell activation. When this hypothesis is true, CCL18 might play an active role in the chronic phase of RA. In that case, serum CCL18 will more directly reflect disease activity.

Little is known on the kinetics and dynamics of CCL18 *in vivo*. Since in our inception cohort both CCL18 levels and clinical data were measured every 3 months, this relati-

vely long time between two measurements might influence the strength of the correlation between CCL18 and DAS-28 over time. The fact that we observed the strongest correlation within the first two weeks after treatment initiation in the anti-TNF-α cohort may support this. However, the decrease in correlation strength over time may also be caused by the initiation of a new treatment regimen. Effects of DMARDs on immune cells are poorly understood, but may result in altered secretion of cytokines and/or chemokines. As a result, this may influence the reflection of the disease course by soluble mediators such as CCL18, which might explain the decrease in correlation strength over time. Next to the time between observations, another possible confounding factor is that elevated CCL18 levels may not only reflect disease activity in the joints, but also organ involvement or co-morbidity. In systemic sclerosis (SSc) for instance, a first indication for a correlation between CCL18 and pulmonary fibrosis has recently been described [38]. Pulmonary fibrosis is a clinical feature that is also known to occur in RA and therefore might be an additional source of CCL18 levels in certain patients. However, of the patients included, only 5 patients had pulmonary co-morbidity with no case of recorded pulmonary fibrosis (data not shown).

The role of TNF-α in the regulation of CCL18 is still unclear. TNF-α blockade decreased CCL18 mRNA expression *in vitro* when administered to DC cultures during maturation, but administration of TNF-α to monocytes did not result in an enhanced secretion of CCL18 [11]. After an initial decrease in CCL18 levels in 95% of the patients, accompanied with a clear correlation with DAS-28, we did not observe a significant correlation between changes in CCL18 and DAS-28 after 14 weeks in our anti-TNF-α cohort. One could speculate that the start of anti-TNF-α treatment initially results in a decrease in CCL18 levels due to a direct effect of TNF-α blockade. In a later stage, the immune system has to search for a new equilibrium without the presence of TNF-α, which could explain why CCL18 levels do not remain low in all patients. The effects of these changes in the immune system may overrule the effect of the changes in disease activity on CCL18 levels. Thus, CCL18 levels might also be affected by the type of treatment, independently of disease activity.

We and others demonstrated that CXCL16 levels are particularly high in RA synovial fluid [9, 39], which is in concordance with the high expression of CXCL16 in RA synovial tissue [9, 39]. CXCL16 levels were not elevated in RA serum at baseline compared to healthy controls and we did not find a significant correlation with clinical disease parameters. This suggests that serum CXCL16 is not useful as a clinical marker in RA. However, this does not imply that CXCL16 does not play a role in the pathogenesis of RA. In the first place, soluble CXCL16 does not represent the total CXCL16 expression, since another significant portion is still membrane-bound. In order to draw conclusions with regard to a role for CXCL16 in the pathogenesis, also mem-

brane bound CXCL16 and the levels of proteases such as ADAM-10 should be taken into account. Second, the data that are currently available on the role of CXCL16 and its receptor CXCR6 in RA point towards a role in local synovial inflammation and are not suggestive for a role in systemic inflammation. Circulating levels of inflammatory mediators do not necessarily reflect local expression in the tissues. For example, circulating levels of TNF-α hardly point towards a significant role in RA, which in fact is well appreciated in daily clinical practice. Given the data from the present study, it appears that circulating CXCL16 levels, not being elevated in RA and without correlation with clinical parameters, do not reflect high local levels in the joints. With regard to the relation between TNF-α and CXCL-16, it is interesting that CXCL16 levels decrease significantly upon treatment with anti-TNF-α, without a significant correlation with disease activity, which was different from our previous observations in the synovial architecture . Whether the decrease in circulating CXCL16 levels upon neutralization of TNF-α is a direct or indirect effect remains to be elucidated. The exact regulation of CXCL16, which may provide more insights in the role of CXCL16 in RA and may help to explain this finding is currently under our investigation.

In summary, we show that elevated CCL18 levels correlate positively with disease activity but not joint damage in RA. This correlation may reflect a role in RA pathogenesis, which might be pro- or anti-inflammatory. CXCL16 levels are not elevated and do not correlate with disease activity or joint damage. Both CCL18 and CXCL16 levels decreased upon treatment with anti-TNF-α, independently of disease activity. Although the strength of the correlations needs to be determined in large studies, our data do not directly support CCL18 as a novel clinical marker in RA, as correlations with disease activity were lower than those of markers as ESR and CRP. More knowledge on the role and regulation of both CCL18 and CXCL16 is needed to value their role in the pathogenesis of RA, both locally and systemically.

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chapter 7a

CAN CXCL16 BE LINKED TO CORONARY VASCULAR DISEASE?

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Letter

With great interest we read the recent paper entitled "Decreased plasma CXCL16/SR-PSOX concentration is associated with coronary artery disease" by Sheikine *et al* [1]. In this study, the authors investigated the relationship between plasma CXCL16 levels and coronary artery diseases (CAD) in a group of healthy individuals and patients with different types of CAD. They found that patients with different CAD exhibit lower systemic CXCL16 levels compared with controls, while CXCL16 levels are not related to the degree of coronary artery stenosis or to biochemical risk indicators of CAD. Based on these data, the authors hypothesize that soluble CXCL16 acts as a scavenger for oxidized LDL (oxLDL) and therefore might have an atheroprotective role.

CXCL16 is a trans-membrane molecule that was first identified as a scavenger receptor for oxidized LDL (oxLDL) [2]. It was shown to play a role in the internalization of oxLDL particles, leading to subsequent foam cell formation, which is a critical step in the development of atherosclerotic plaques. Upon cleavage by proteases, soluble CXCL16 exerts its effect as a chemokine ligand for CXCR6+ cells [3, 4]. Importantly, enhanced expression of membrane-bound CXCL16 has been found in atherosclerotic plaques, suggesting its role in the pathogenesis of atherogenesis [5]. Therefore, the assessment of a link between CXCL16 and cardiovascular disease is justified. However, there are some concerns regarding the findings and interpretations of Sheikine and co-workers.

First, plasma CXCL16 levels were only significantly decreased in patients with stable angina and not in patients with unstable angina, a more severe coronary syndrome associated with increased plaque activity (fig 1a of Sheikine *et al*). A high inter-person variability of the CXCL16 plasma concentrations and small groups, which might decrease the power of analyzed data, were stated as possible explanations for this discrepancy. However, in an almost ten times larger group no significant differences could be seen between patients with a recent myocardial infarction and healthy controls (fig 1b of Sheikine *et al*). Therefore, the results that were obtained comparing the stable angina patients with the healthy control group, on which the authors

based their hypothesis, become controversial. Perhaps the relation between the onset of CAD symptoms and the moment when CXCL16 measurements were performed might be of interest, since decreased levels are found after three days in the stable and unstable angina patients while this is no longer present after three months in patients with myocardial infarction. This however was not investigated.

Second, the interpretation of the results by the authors suggests that the soluble form of CXCL16 can bind oxLDL. This might be true since CXCL16/SR-PSOX exerts its scavenger role through its chemokine domain [6], which is present in both membrane bound and cleaved CXCL16. However, a scavenger effect has thus far solely been demonstrated for membrane bound CXCL16/SR-PSOX. The authors offer no data to demonstrate the effect of a direct interaction between soluble CXCL16 and ox-LDL, which makes the hypothesis that less circulating CXCL16 is associated with less oxLDL scavenging debatable. One could propose that decreased circulating soluble CXCL16 levels reflects an elevated expression of membrane-bound CXCL16/SR-PSOX, which may contribute to enhanced uptake of LDL and foam cell development. However, it has never been shown that enhanced cellular expression of CXCL16 is correlated with lower levels of soluble CXCL16. This would be dependent on activity of proteases such as ADAM-10, which has not been taken into account by the authors. Furthermore, it has never been shown that activity of ADAM-10 in terms of CXCL16 cleavage would be different in pathological conditions. In addition, the authors have assessed no other scavenging systems for oxLDL, which might also have an influence on the development of atherosclerotic plaques.

Despite these caveats, the study by Sheikine and colleagues suggests a potentially interesting link between CXCL16 and cardiovascular risk. This might also be of interest in chronic inflammatory conditions, in which a role for CXCL16 in the pathogenesis has been suggested. Interestingly, patients with chronic inflammatory conditions have a significantly increased incidence of cardiovascular disease and related morbidity and mortality. In fact, patients with rheumatoid arthritis have an increased risk for developing CAD, which is independent of disease activity [7]. Intriguingly, CXCL16 was recently found to be increased in synovial fluid and tissue of RA patients [8, 9]. The present study by Sheikine et al contributes to the discussion whether CXCL16 can be accounted for this increased cardiovascular risk in chronic inflammation, which is currently under our investigation.

Altogether, we believe the conclusions by Sheikine *et al* are preliminary and the relevance of their data can only be valued when a role for cleaved CXCL16 in scavenging oxLDL is demonstrated and the current data are confirmed in larger cohorts, in particular cohorts with more patients with stable and/or unstable angina. The relation

between CXCL16 and CAD is certainly intriguing and the paper by Sheikine *et al* emphasizes the complexity of this interesting molecule and encourages thorough research on its role in pathological processes.

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chapter 7b

CIRCULATING CXCL16 IS NOT RELATED TO CIRCULATING OXLDL IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Abstract

CXCL16 acts as a scavenger receptor for oxLDL in its membrane-bound form and induces migration of activated T cells in its soluble form. Due to these properties, CXCL16 has been suggested to play a role in both atherosclerosis and rheumatoid arthritis (RA). Our aim was to evaluate the contribution of soluble CXCL16 to the scavenging of oxLDL and its potential as a marker for cardiovascular disease (CVD) in patients with RA. We found that circulating CXCL16 was not correlated with plasma oxLDL or ApoB and was not related to the presence of CVD in RA patients. Moreover, CXCL16 did not bind and scavenge oxLDL in an *in vitro* setting. These data suggest that binding of oxLDL by soluble CXCL16 does not play a role in atherosclerosis and, although confirmation in larger studies is needed, that circulating CXCL16 is not related to the presence of CVD in patients with RA.

Introduction

Atherosclerosis is one of the hallmarks of cardiovascular disease (CVD). Although the exact processes that drive atherosclerosis remain to be identified, there is substantial evidence to support the concept that atherosclerosis is an inflammatory process [1]. Infiltration by leucocytes, such as T lymphocytes and monocytes, is one of the key features in the formation of atherosclerotic plaques [2]. Monocytes that are attracted to the vessel wall differentiate into activated macrophages, which subsequently can transform into cholesterol loaded foam cells [3]. Oxidation of LDL and internalization of oxidized LDL (oxLDL) are considered to be the initiating events in this foam cell formation [4, 5] and are therefore critical processes in the early development of atherosclerosis. Circulating oxLDL has been shown to be useful as a marker for identifying patients with coronary vascular disease [6-10]. In addition, plasma oxLDL strongly correlates with other predictors of enhanced cardiovascular risk, including LDL-cholesterol and apolipoprotein B (ApoB) [11-13].

In order to internalize oxLDL, activated macrophages make use of scavenger receptors that are situated on their surface [3]. CXC Chemokine Ligand 16 (CXCL16) is a trans-membrane molecule that was shown to act as such a scavenger receptor for oxLDL [14]. Besides on macrophages and dendritic cells [15], CXCL16 is expressed on the surface of smooth muscle cells [16], which also take part in the atherosclerotic plaque development process. Interestingly, enhanced expression of membrane-bound CXCL16 has been found in atherosclerotic plaques, substantiating its potential role in the pathogenesis of atherosclerosis [17]. Next to its membrane-bound form, CXCL16 can be shed from the cell surface upon proteolytic cleavage [18] to act as a chemokine ligand for CXCR6+ cells [19]. This receptor is present on activated/memory type T cells, plasma cells and NKT cells [19-21]. Recently it has been suggested that decreased circulating CXCL16 levels might reflect an increased risk for CVD and may constitute a novel marker for the assessment of cardiovascular risk [22].

Rheumatoid arthritis (RA) is a chronic inflammatory disease with an increased morbidity and mortality due to CVD compared with the general population [23]. In line with this increased prevalence of CVD, RA patients have an increased burden of cardiovascular risk factors, including an atherogenic lipid pattern with lower HDL-cholesterol and elevated plasma oxLDL concentrations [24], altered insulin sensitivity [25] and increased plasma concentrations of pro-inflammatory markers, including CRP, IL-6 and TNF [26]. In RA, CXCR6 is highly expressed on synovial fluid T cells [27-29]. Furthermore, we recently described the abundant expression of CXCL16 in the synovial compartment in RA, suggesting a role for CXCL16 in the pathogenesis of RA [29], which was later confirmed by other groups [28, 30].

Despite the evidence that supports a role for CXCL16 in atherosclerosis, the potential role of its soluble variant in atherosclerosis is unclear, as is the possible contribution

of soluble CXCL16 to the increased cardiovascular risk in RA. The aim of our study was, therefore, to investigate whether circulating CXCL16 levels reflect this increased risk and might compete with membrane-bound CXCL16 -mediated scavenging of oxLDL by binding to oxLDL in a liquid phase. Here, we provide data that question a role for soluble CXCL16 in the process of oxLDL internalization and its use in cardiovascular risk assessment.

Methods

Patients and samples

RA patients were recruited from the Nijmegen inception cohort, which is an ongoing cohort that continuously includes patients with early RA (disease duration <1 year and no prior use of disease-modifying anti-rheumatic drugs (DMARDs). For the present analysis, thirty consecutively enrolled patients with at least 2 years of follow-up data were selected. During the study period, a variety of demographical data were recorded, including DMARD therapy and cardiovascular co-morbidities, which in this case were myocardial infarction, angina pectoris and primary hypertension (Table 1). Serum samples were taken at baseline and every three months during the follow-up period. OxLDL, ApoB, CXCL16 and other inflammatory markers, including CRP and ESR, were determined in these samples. In addition, disease activity was assessed at each of the above-mentioned time-points using the DAS28 score [31].

Biochemical analyses

CXCL16 levels were measured with a sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) as described previously [29, 32]. The detection limit of the ELISA is 100 pg/ml. All samples were measured in only two separate assays. For oxLDL measurements a commercially available competitive ELISA (Mercodia, Uppsala, Sweden) was used and performed according to the manufacturers' instructions. Plasma ApoB levels were determined with ELISA using polyclonal IgG antibodies raised by ourselves in a goat. The antibody is also used for immunonefelometric measurement of plasma apolipoprotein B and both assays showed high correlation (r=0.97, n=72; data not shown).

CXCL16-oxLDL co-immunoprecipitation

To test whether soluble CXCL16 can bind oxLDL in a liquid phase we performed a co-immunoprecipitation assay. First, cobalt beads (BD Biosciences) were washed 3x in PBS and transferred into tubes that were pre-coated with 1% BSA in PBS. Next, histidine tagged recombinant human CXCL16 (rhCXCL16-His) (R&D systems) was incubated with the beads for 2 hours at 4°C in four rising concentrations, all in triplicate. As a negative control, beads without rhCXCL16 were taken along. There-

Table 1. Patient characteristics at baseline.

Patient characteristics	Mean ± SD
General characteristics	
Age (years)	52 ± 15
Gender (female/male)	23/7
RF presence (%)	72
DAS28	6.0 ± 1.2
CXCL16 (pg/ml)	4669 ± 1442
oxLDL (U/l)	36.6 ± 15.4
apoB (mg/l)	451 ± 197
Cardiovascular co-morbidity	
MI (%)	10
HT(%)	10
AP(%)	0

Results are expressed as Mean ± standard deviation (SD) unless stated otherwise; RF = rheumatoid factor; oxLDL = oxidized LDL; ApoB = apolipoprotein B; MI = myocardial infarction; HT = hypertension; AP = angina pectoris

after, the beads were centrifuged and the supernatant was frozen for later CXCL16 measurements. After 3 wash steps with PBS, the beads were incubated with a fixed concentration of 76mU/L oxLDL (Mercodia, Uppsala, Sweden) overnight at 4°C. To pull down the beads, the solution was centrifuged and oxLDL still present in the supernatant was determined with ELISA as described above. To test whether there was still rhCXCL16-His bound to the beads, the beads were washed, eluted with 10mM EDTA in PBS and centrifuged. Finally, eluted CXCL16 was measured in the supernatant of the eluted beads as described above.

Statistical analysis

Within our group of RA patients, comparisons between different time-points during the follow-up period were made using the Wilcoxon signed rank test for non-parametric values, while the paired Student's t-test was use in the case the values were normally distributed. A Mann-Whitney test was used to make the comparisons between RA patients with and without CVD. Correlations between CXCL16 and lipid markers were determined using the Spearman's test. p-values <0.05 were considered significant. Values are expressed as mean (+ standard deviation (SD)), unless stated otherwise.

Results

Patient characteristics

Patient characteristics at baseline are depicted in table 1. Of all patients, 20% (6/30) had cardiovascular co-morbidity before they were enrolled in the study, which was either a myocardial infarction (10%, 3/30) or hypertension (10%, 3/30). Notably, all 3 patients who previously experienced a myocardial infarction were male. During the follow-up period, we recorded 2 more cases of ischemic cardiac disease, being one angina pectoris in a patient with history of hypertension and one myocardial infarction. The therapeutic regimen of our patients during the study period comprised at least one DMARD in 93% of the patients, while only two patients did not receive DMARD treatment during this interval. Disease activity significantly declined from a DAS-28 score of 6.0 ± 1.2 at baseline to 3.9 ± 1.2 two years after starting anti-rheumatic therapy ($p<0.0001$).

Circulating CXCL16 levels do not correlate with CVD markers in RA patients

As mentioned above, oxLDL and ApoB are considered to be important markers of cardiovascular risk [11-13]. To assess the possible relation between soluble CXCL16 and these markers, we measured circulating levels of these molecules in all patients at baseline (table 1) and throughout the follow-up period. The mean CXCL16 level was 4669 ± 1442 pg/ml, which is comparable with results that we found in large panel of healthy controls and RA patients [33]. In contrast to circulating CXCL16 levels, which remained similar to baseline, plasma concentrations of oxLDL and ApoB increased during the first two years after initiation of conventional anti-rheumatic therapy (data not shown). However, the ratio oxLDL/ApoB significantly decreased from 0.09 ± 0.03 U/mg to 0.06 ± 0.01 U/mg ($p<0.002$) during the same interval. Soluble CXCL16 levels neither correlated with plasma oxLDL, nor with plasma ApoB at any of the investigated time points (table 2). In contrast, oxLDL and ApoB levels were significantly correlated during the entire follow-up period (table 2). Furthermore, CXCL16 levels were not different in patients with a history of CVD compared to non-CVD patients, irrespective to the time-point (table 3). Although plasma oxLDL and ApoB levels were not significantly different between RA patients with and without history of CVD, both variables were clearly higher in RA patients with CVD (table 3).

Soluble CXCL16 does not bind oxLDL in vitro

Membrane-bound CXCL16 is known to effectively bind oxLDL [14]. The contribution of soluble CXCL16 has been discussed in the literature [34, 35] but is still unclear. Therefore, we questioned whether CXCL16 is able to bind oxLDL in a liquid phase. In figure 1a, we show that incubation of oxLDL with increasing concentrations of rhCXCL16-His bound to cobalt beads did not result in a decrease in oxLDL concentrations, indicating that oxLDL does not stably bind to soluble CXCL16. To make sure

Table 2. Correlations between CXCL16, oxLDL and apoB concentrations at different time-points during the study period.

Variables	Time				
	baseline	6 months	12 months	18 months	24 months
CXCL16 and oxLDL	r = -0.16 p = 0.42	r = -0.13 p = 0.56	r = -0.08 p = 0.69	r = -0.04 p = 0.85	r = -0.30 p = 0.14
CXCL16 and apoB	r = -0.28 p = 0.17	r = -0.06 p = 0.78	r = -0.01 p = 0.98	r = -0.07 p = 0.74	r = -0.39 p = 0.06
oxLDL and apoB	r = 0.81 p < 0.001	r = 0.65 p < 0.001	r = 0.84 p < 0.001	r = 0.81 p < 0.001	r = 0.89 p < 0.001

Spearman correlations. r = correlation coefficient; p = significance value; oxLDL = oxidized LDL; apoB = apolipoprotein B

Table 3. Circulating concentrations of CXCL16, oxLDL and apoB in RA patients with and without CVD.

Variables		Time				
		baseline	6 months	12 months	18 months	24 months
oxLDL (U/l)	CVD	43.1 ± 17.0	43.0 ± 14.1	62.1 ± 53.7	60.8 ± 27.9	50.9 ± 12.7
	non-CVD	34.6 ± 14.7	33.7 ± 11.0	40.7 ± 17.8	41.9 ± 13.9	41.9 ± 14.5
		p = 0.29	p =0.19	p = 0.38	p = 0.10	p = 0.13
apoB (mg/l)	CVD	507 ± 214	639 ± 224	761 ± 260	925 ± 335	820 ± 293
	non-CVD	434 ± 193	539 ± 258	631 ± 194	680 ± 196	667 ± 182
		p = 0.53	p = 0.31	p = 0.35	p = 0.10	p = 0.18
CXCL16 (pg/ml)	CVD	4746 ± 1532	5033 ± 1331	5287 ± 1418	5327 ± 2854	4694 ± 3298
	non-CVD	4647 ± 1454	5029 ± 1876	4597 ± 1572	4904 ± 1586	4256 ± 1269
		p = 0.82	p = 0.97	p = 0.34	p = 0.89	p = 0.33

Mann-Withney test was used to make the comparisons between CVD and non-CVD RA patients at each time-point for each parameter studied. Results are expressed as mean ± standard deviation; RA = rheumatoid arthritis; CVD = cardiovascular disease; oxLDL = oxidized LDL; apoB = apolipoprotein B.

CXCL16 was effectively bound to the beads, we measured CXCL16 directly after incubation. This binding was highly effective, since 97-99% of the total CXCL16 input was bound to the beads (figure 1b). Importantly, also after incubation with oxLDL and several wash-steps, we could effectively elute CXCL16 from the beads in a clear concentration course (figure 1c), indicating that a significant amount of CXCL16 had

been present throughout the assay. In order to exclude that the lack of binding could be explained by the temperature at which the assay was performed, we repeated the experiment at 37° C, which resulted in similar results (data not shown).

Discussion

In the present study, we showed that circulating CXCL16 levels do not correlate with oxLDL levels and are not related to the presence of CVD in patients with RA. The lack of a correlation between oxLDL and CXCL16 was further substantiated by the inability of CXCL16 to stably bind to oxLDL in an *in vitro* setting. Together, these data question a direct contribution of soluble CXCL16 by binding oxLDL to the process of atherosclerotic plaque development.

Based on its potential to act as a scavenging receptor for oxLDL [14] and its enhanced expression in atherosclerotic plaques [17, 36], CXCL16 was suggested to play a role in atherosclerosis. However, there are still several major gaps in our knowledge on the role of CXCL16 in foam cell formation. First, the relative contribution of CXCL16 to the oxLDL scavenging process *in vivo* is uncertain, as is the exact consequence of this oxLDL binding. Second, the contribution of CXCR6+ T cell attraction to the atherosclerotic process is insufficiently investigated. Finally, it is unclear whether soluble CXCL16 might play a role in the balance of oxLDL uptake and degradation. Next to fractalkine (CX3CR1), CXCL16 is one of the two known chemokines that exist in a trans-membrane form. It has been shown that CXCL16 binds oxLDL through its chemokine domain [37], which is located in the extra-cellular part of the molecule. One could, therefore, hypothesize that binding of oxLDL to soluble CXCL16 might result in a competition with cell surface scavenger receptors and subsequently in less oxLDL internalization and foam cell formation. Interestingly, decreased circulating CXCL16 levels were recently proposed to be associated with increased cardiovascular risk and consequently plasma CXCL16 was suggested to be atheroprotective [22]. This concept, however, is not supported by the data from the present study, since we showed that CXCL16 does not correlate with oxLDL *in vivo* and does not bind oxLDL *in vitro*. Moreover, we found no differences in circulating CXCL16 levels between RA patients with and without cardiovascular co-morbidities. To draw firm conclusions on this relation, this finding should be confirmed in a larger cohort with individuals that do not have RA as a concomitant disease, since both RA and the small number of patients evaluated in the present study may influence the relation between CXCL16 levels and CVD. However, we and others have recently shown that serum CXCL16 levels in RA are not significantly different from those in healthy controls [28 33]. This implicates that our results cannot be explained by altered CXCL16 levels in RA that may bias the correlation between CXCL16 and oxLDL. Thus based on our results, decreased CXCL16 levels do not appear to be correlated with CVD.

Recently, other evidence supporting an atheroprotective role for CXCL16 came from experiments using murine models [38]. Accordingly, it was shown that CXCL16 and LDL receptor double knockout mice suffered from an accelerated development of atherosclerosis as compared with LDL receptor -/- mice. This was highly intriguing, since macrophages from CXCL16 -/- mice internalized significantly less oxLDL compared to wild-type macrophages, which suggests that oxLDL binding by CXCL16 might not result in typical foam cell formation that favours atherosclerosis. Interes-

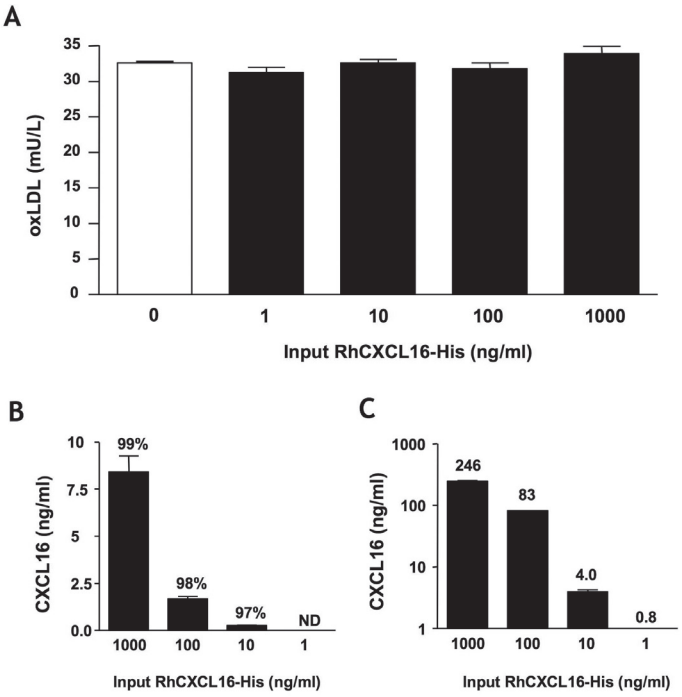

Figure 1:

Recombinant human CXCL16 does not bind oxLDL *in vitro*

Panel A represents the amount of oxLDL that was recovered in the supernatants of solutions of oxLDL (76 mU/L) after incubation with CXCL16 coated beads and subsequent pull-down. Beads coated with increasing concentrations of CXCL16 were incubated with a stable concentration of oxLDL (76 mU/l). Absolute values are given on top of the bars. The bars represent mean and SEM of triplicates and the figure represents one of two individual experiments. *Panel B* shows the efficacy of the binding of rhCXCL16-His to cobalt beads. The bars represent the concentration of CXCL16 that was measured in the supernatant of the beads solution after centrifugation and the percentage of effective binding is shown on top of the bars. The bars represent mean and SEM of one out of two experiments performed in triplicate. CXCL16 measurements were done in duplicate. ND = Not detectable.

Panel C shows the concentration of CXCL16 that was eluted from the cobalt beads after incubation with oxLDL. The bars represent mean and SEM of one out of two experiments performed in triplicate. CXCL16 measurements were done in duplicate.

tingly, 80% more apoptosis was found among CXCL16 -/- macrophages compared to wild-type macrophages, which might be an explanation for accelerated atherosclerosis, but needs to be further investigated. Studies comparing CXCL16 -/- macrophages with macrophages deficient of other scavenger receptors may reveal whether processing of oxLDL via CXCL16 is atheroprotective. Another explanation for accelerated atherosclerosis in CXCL16 -/- LDL receptor -/- mice might have been the lack of soluble CXCL16 as a competitive binder of oxLDL. However, given the data from the present study, this is rather unlikely.

In summary, our study does not advocate a role for circulating CXCL16 levels as a marker for the atherosclerosis and/or subsequent cardiovascular disease in RA. Whether the membrane-bound form of CXCL16 and its ability to internalize oxLDL acts pro- or anti- atherogenic remains the major question to be answered.

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chapter 8

FINAL CONSIDERATIONS

Chemokines orchestrate leukocyte migration. For this reason they play a pivotal role in immune responses in general and in inflammatory diseases such as RA. APC and T cells are two groups of leukocytes which are both crucial players in immunity and tolerance and hence in various auto-immune diseases. Chemokines are instrumental in establishing the interaction between leukocytes, including APC/T lymphocyte interactions. Herein, we explored the potential role of two relatively novel chemokines that are secreted by APC to attract T lymphocytes: CCL18 and CXCL16. In chapter 2 t/m 7, we provided novel data on the potential role and regulation of these chemokines in general and in RA.

CCL18

The chemokine CCL18 is expressed and secreted by myeloid cells such as DC and macrophages. In chapter 3, we showed that resting monocytes do not secrete significant amounts of CCL18 protein [1]. However, when stimulated with the Th2 cytokines IL-4 or IL-13, they rapidly secrete CCL18. Therefore, it seems that CCL18 secretion by myeloid cells is induced by IL-4 or IL-13, although these cytokine effects cannot be easily separated from their role in cell differentiation, e.g. the generation of MoDC in the presence of IL-4 and GM-CSF. Based on our results from chapter 2 and 3, there seems to be some discrepancy between regulation on mRNA and protein level. In contrast to CCL18 protein secretion (chapter 3) [1], CCL18 mRNA expression is upregulated upon maturation, as shown in chapter 2 [2]. As for DC maturation, the effect of TNF-α on CCL18 expression and secretion also appear to be inconsistent. In chapter 2, we showed that the strong increase in CCL18 mRNA upon DC maturation could be inhibited by neutralizing endogenous TNF-α, independently of the maturation state [2]. However, in chapter 3 we found that stimulation of monocytes/macrophages with TNF-α did not sort any effect on CCL18 protein secretion [1]. The difference between CCL18 mRNA and protein might suggest that CCL18 secretion can be further upregulated by additional stimuli, which are yet unknown. Recent data from Auer and colleagues on CCL18 expression and secretion by neutrophils underscore this distinct regulation on mRNA and protein level [3]. In this study, TNF-α also indu-

ced CCL18 mRNA but not protein. For protein release, an additional stimulus, e.g. IL-10, was needed. Interestingly, they too found evidence suggesting an additional CCL18 inducing factor in RA joints, in line with our data from chapter 3 [1]. Detailed studies on CCL18 mRNA vs. protein regulation would be of interest to solve this part of the CCL18 regulation puzzle. As DC maturation by itself did not sort any effect on CCL18 secretion by MoDC, it is not surprising that we did not observe effects on CCL18 secretion on protein level upon blocking TNF-α during maturation (van Lieshout *et al*, unpublished observations). The direct effects of TNF-α on CCL18 secretion *in vitro* therefore seem to be limited. *In vivo* however, we did observe a significant decrease in circulating CCL18 levels upon neutralizing TNF-α in patients with RA, as shown in chapter 6 [4]. However, it would be preliminary to conclude that this comprises a direct effect of TNF-α blockade, as TNF-α is a pleitropic cytokine affecting multiple direct and indirect pathways. For instance, anti-TNF-α was recently associated with the induction of a distinct population of regulatory T cell [5] and altered DC phenotype [6], which might have an effect on circulating cytokine and/or chemokine levels. Perhaps *in vivo* the effect of TNF-α on CCL18 expression is translated from mRNA to protein due to the effect other mediators which are not present *in vitro*, leading to the apparent contrast between *in vivo* and *in vitro* regarding the effect of TNF-α on CCL18 protein. Taken together, TNF-α may have its influence on CCL18 through effects on mRNA production, but pathways leading to effects on protein secretion are yet to be elucidated.

The anti-inflammatory cytokine IL-10 proved to be a strong inducer of CCL18 protein secretion. Interestingly, IL-10 is a well appreciated inhibitor of DC maturation [7]. Since DC maturation itself did not sort any effect on CCL18 secretion, these data suggest that IL-10 acts as a direct inducer of CCL18. Moreover, IL-10 also induced CCL18 secretion by monocytes and not only DC. Perhaps the most intriguing observation is that IL-10 acts in strong synergy with IL-4 and IL-13. These synergistic effects of IL-10 on CCL18 secretion by monocytes/macrophages explain the large amounts of secreted CCL18 by MoDC that were stimulated with IL-10, as these cells were already primed with IL-4. As IL-4 and IL-13 are key players in the regulation of CCL18, CCL18 is well suitable as a marker for "alternative activation" of myeloid cells, which is now commonly accepted in literature [8-10]. Although the effects of IL-4/IL-13 and IL-10 on CCL18 secretion are unmistakable, it would be preliminary to conclude that these are "THE" CCL18 inducing agents. For one, it is still unknown which intracellular signaling cascades drive CCL18 secretion. Elucidation of these cascades would help to identify novel mediators that drive CCL18 protein secretion. Furthermore, this might help to unravel which mediators present in RA SF cause the strong synergistic effects we showed in chapter 3 [1]. More knowledge on CCL18 regulation will ultimately lead to an increased understanding of the physiological role

of this chemokine, CCL18 levels are elevated in serum (chapter 6) and synovial fluid of RA patients [4, 11], its mRNA expression level by MoDC is elevated in RA (chapter 2) [2, 12] and protein expression is enhanced in RA ST (chapter 3) [1, 12]. The most obvious explanation for elevated circulating CCL18 levels in RA would be an enhanced CCL18 secretion by myeloid cells. In contrast to mRNA [2, 12], we did not detect significant differences in CCL18 protein secretion between mature DC from RA patients and healthy controls *in vitro*. [1]. This may indicate that an additional trigger is needed to induce enhanced CCL18 protein secretion in RA, as already suggested above. In this light, the synergistic effects of RA SF on CCL18 (chapter 3) are intriguing [1], as this SF may contain such an additional factor. This then is likely to be a local factor in RA joints, as pre-incubation *in vitro* with RA SF enhanced CCL18 secretion, whereas freshly RA blood monocytes do not secrete more CCL18 upon stimulation than monocytes that have not encountered a RA environment *in vivo*. Intriguingly, this "imprinting" phenomenon in RA joints was recently also suggested as an explanation for high CCL18 secretion by neutrophils isolated from RA joints [3]. Another explanation for enhanced CCL18 expression in RA may be increased amounts of CCL18 secreting cells, although elevated circulating leukocyte counts are not typical for RA. Finally, elevated levels of IL-10, which have been reported in literature [13], or IL-4 or IL-13 may contribute to elevated CCL18 levels in RA. Whether a causal relation exists between levels of these cytokines and CCL18 levels however is difficult to investigate in the *in vivo* setting.

As discussed above, the functional role of CCL18 in humans is still largely unknown and novel insights in its functions *in vivo* are only reported sporadically. For one, the lack of a murine CCL18 homologue and has prevented relatively straight forward murine studies on the functional role of CCL18 *in vivo*. Interestingly, some groups have investigated the effects of human CCL18 in mice. Bruna-Romero and colleagues used an adenoviral vector to over-express human CCL18 in a murine malaria model [14] and Pochetuhen and colleagues examined the effects of human CCL18 in a murine model of pulmonary fibrosis [15]. These experiments are all based on the assumption that a yet unknown receptor for CCL18 does exist in mice. Although these studies have claimed some effects of CCL18, without clear evidence for the existence of a CCL18 receptor these data should be regarded as anecdotal. In a pilot study, we could not observe any chemotactic effect of intra-articular injection of ad-CCL18 into murine knee joints, despite the fact that significant amounts of CCL18 protein could be detected in the joints (unpublished observations). Perhaps models using macaques could provide additional information on the role of CCL18 *in vivo*, as has been suggested previously [16]. Alternatively, humanized-mouse models could be explored. Besides the lack of a murine variant, CCL18 research is hampered by the fact that a high affinity receptor is still unknown. Identification of either a high

affinity receptor or (multiple) low affinity receptor(s) will lead to crucial new tools for CCL18 research, e.g. blocking studies to gather more precise data on its chemotactic properties. Moreover, detecting its target cells *in vivo* would help to identify the role of CCL18 in inflammation. Reviewing the currently available literature, the question whether CCL18 acts pro- or anti-inflammatory *in vivo* remains unanswered. In general, CCL18 is well appreciated for its role as a chemotactic agent for Th0 cells [17]. In addition, it has been shown that CCL18 may also attract other T cell subsets and germinal centre B cells under certain circumstances [18-21]. The ability to attract these leukocytes and its secretion by APC suggests that CCL18 plays a role in the initiation of immune responses. Its *in vivo* expression in germinal centers and lymphoid structures is in line with this hypothesis. However, it is unknown what the exact contribution of CCL18 is to T cell attraction, in comparison to other Th0 cell attraction chemokines such as CCL19. In RA, strategic CCL18 expression in the ST and its chemotactic properties may result in attraction of Th0 cells towards the site of inflammation. As the factors that drive CCL18 secretion are mostly anti-inflammatory, this would imply that CCL18 secretion in RA inflamed RA joints is intended as an anti-inflammatory response. However, it is unclear whether the eventual result of Th0 influxes in RA joints is pro- or anti-inflammatory, since this is dependent on the differentiation of Th0 cells. Th0 cells can differentiate into Tregs or go into apoptosis upon contact with APC, resulting in an anti-inflammatory response. In contrast, the inflamed RA environment, with its abundance of pro-inflammatory cytokines, may also result in differentiation of Th0 cells into activated Th1-like cells, resulting in a pro- in stead of anti-inflammatory response.

Despite the uncertainty of the eventual effect of CCL18 secretion in RA, its circulating levels may correlate with clinical disease activity scores, which we demonstrated in chapter 6 [4]. Although CCL18 significantly correlated with disease activity, this correlation was rather low. Thus, CCL18 has little or no additional value as a disease marker over the existing ones, such as the DAS-28 and its individual constituents. Moreover, elevated CCL18 levels are not exclusive for RA but have now been described in a variety of different auto-immune diseases [22-27], amongst others SSc [28, 29] and SLE [30]. Especially in SSc, the potential impact of CCL18 might go further than in RA. First, its pro-fibrotic effects [31] make this chemokine a highly interesting subject of investigation in SSc, a disease in which fibrosis is one of the hallmarks. Second, CCL18 may be a downstream player in this disease in which expression of Th2 cytokines is enhanced [32]. In turn, CCL18 may contribute to the disease process through T cell attraction. Recently, CCL18 levels were shown to be elevated in patients with SSc by independent groups [28, 29]. Interestingly, CCL18 has indeed been suggested as a novel clinical marker for pulmonary fibrosis in SSc [28, 29]. Large prospective cohorts are however needed to validate the potential of CCL18 as a clinical marker for disease

activity in SSc and further experimental studies will have to determine the exact role of CCL18 in the pathogenesis of SSc, as is the case for RA.

Taken together, the currently available data are at least suggestive for some involvement of CCL18 in the complex pathogenesis of RA. However, as yet, any suggestion regarding the use of CCL18 as a therapeutic target in RA would be preliminary. Further research on CCL18 and other chemokines is essential to determine the importance of CCL18 within the "chemokine world" in general, and in RA pathogenesis and treatment in particular.

CXCL16

The T cell attracting chemokine CXCL16 is now believed to play a role in a variety of inflammatory conditions. Although CXCL16 is indisputably secreted by myeloid cells, the triggers that induce this secretion are still largely unknown. In chapter 5 and 6, we provided novel insights in the regulation of CXCL16 expression and secretion by myeloid cells, both in general and in RA [33, 34]. Interestingly, simply culturing monocytes proved to be the strongest trigger for CXCL16 upregulation *in vitro*. As monocytes differentiate into macrophages or DC *in vitro*, this suggests that monocytes may upregulate CXCL16 expression when entering the tissues to differentiate into macrophages or DC. It is therefore tempting to hypothesize that CXCL16, like CC18 [35, 36], may be useful as a marker for tissue residing myeloid subsets *in vivo*. Based on our results from chapter 4 [33], TNF-α seems to act directly on CXCL16 secretion by day 2-3 macrophages. In chapter 5, we show that this effect is absent in freshly isolated monocytes [34]. The explanation for this apparent inconsistency was a decrease in CXCL16 expression in unstimulated myeloid cells after 36-48 hours, which did not take place when cells were stimulated with TNF-α, growth factors or other cytokines (unpublished observations). Moreover, cells that became CXCL16 negative after 48 hours died within 72 hours of culture, in contrast to CXCL16 positive cells. TNF-α therefore does not directly induce CXCL16 expression on monocytes/macrophages, but rather preserves CXCL16 expression compared to unstimulated myeloid cells *in vitro*. In chapter 6, we observed some effect of anti-TNF-α treatment in RA patients on circulating CXCL16 levels, but this effect was inconclusive [4]. As for CCL18, this is unlikely to be a direct effect of TNF-α neutralization.

While monocyte culture or differentiation into DC or macrophages was the strongest trigger to induce CXCL16 expression and secretion, Th1 vs. Th2 polarization also has some effect on CXCL16 expression. In chapter 5, we demonstrated a small effect of IFN-γ on CXCL16 secretion by cultured monocytes *in vitro* [34]. This effect was not observed on CXCL16 membrane expression at 36 hours (data not shown).

Recently, a positive effect of IFN-γ on CXCL16 membrane expression was reported [37]. However, this contrast with our findings is difficult to judge, as different controls were used. Omitting IgG controls may introduce a bias, especially since IFN-γ is well appreciated for its effect on FcR expression. In contrast to the Th1 cytokine IFN-γ, the Th2 and anti-inflammatory cytokines IL-10, IL-4 and IL-13 inhibited CXCL16 secretion to some extend. This effect on CXCL16 secretion is interesting, the more since it is exactly opposite to what is observed for CCL18. *In vivo* studies will have to reveal whether CCL18 and CXCL16 play an important role in typical Th1 or Th2 driven diseases, although these T helper dogma's may lead to over-simplification of the underlying immunological process of inflammatory diseases. Given the high circulating levels of both CXCL16 and CCL18 *in vivo*, it might be interesting to investigate whether these chemokines have opposing functional properties next to opposing regulation. In contrast to Th2 cytokines, DC maturation had little or no effect on CCL18 or CXCL16, while it is well known to induce CCL19, a chemokine that is important for migration of both APC and T cells towards lymph nodes. The fact that tissue resident DC and macrophages secrete large amounts of CXCL16 suggests that CXCL16 might be important for co-localization of these cells and T lymphocytes in tissues, which may induce or maintain inflammatory responses. The elevated local CXCL16 expression in a large variety of inflammatory diseases appears to be in line with this hypothesis. As CXCL16 attracts activated T cells in stead of Th0 cells which can still differentiate for instance Tregs, CXCL16 secretion in inflammatory diseases is likely to have a pro-inflammatory effect.

The first evidence for a role for CXCL16 in RA came when its receptor CXCR6 was found to be highly expressed in RA joints [38]. In chapter 4, we were the first to demonstrate that CXCL16 expression is enhanced in RA ST and able to attract activated T lymphocytes [33]. This again suggests that CXCL16 might play a role in the pathogenesis of RA. As soluble CXCL16 is found in the circulation in large amounts, we hypothesized that this may reflect disease activity in RA in chapter 6. This however was not the case, as CXCL16 levels did not correlate with disease activity [4]. Based on these results, measuring circulating CXCL16 as a diagnostic or disease activity marker for RA has no additive value. This however does not exclude a role for CXCL16 in the pathogenesis of RA. First, we found high CXCL16 expression locally in the ST of RA patients, suggesting that it may play a role in local inflammation. Second, it is debatable whether circulating levels reflect total expression, as a fraction of CXCL16 is membrane-bound and is not measured in serum or plasma. Perhaps local CXCL16 expression in the ST may reflect disease activity, which needs to be examined in studies using synovial biopsies, preferentially in a prospective cohort of RA patients with tight monitoring of clinical disease parameters. As CXCL16 may be an important factor for local synovial inflammation, it's neutralization may be bene-

ficial in RA. However, we are still in the very beginning of CXCL16 research *in vivo*. Nonetheless, the first study which neutralized CXCL16 in murine collagen induced arthritis (CIA) provided encouraging results [39]. These results need to be confirmed in other arthritis models, including CXCL16 -/- or CXCR6 -/- mice. A potential drawback is that it is unknown what the effect of CXCL16 or CXCR6 neutralization might be in terms of physiology. CXCL16 -/- mice are viable, but too little is known about their defence against micro-organisms or predisposition for malignancies. In this light, a local approach might be preferred. However, in clinical practise, techniques aiming to achieve local neutralization in the joint are still a major challenge. Perhaps gene therapy is an interesting method to accomplish such local neutralization in the future. Very recently, a promising study was published on safety and efficacy of gene transfer for Leber's congenital amaurosis, which may encourage investigators to further explore this therapeutic strategy. Next to murine knock-out or blocking studies, another potentially interesting approach to study the functional role of CXCL16 *in vivo* would be to label CXCR6 positive cells to examine their homing *in vivo* in inflammatory conditions. This would allow us to investigate whether influxes of CXCR6+ cells appear prior to clinical signs of joint inflammation, which in turn would plead for CXCL16 or CXCR6 neutralization as a therapeutic strategy in RA. The safety of such an experiment of course needs to be determined in *in vitro* and murine models. However, recently developed techniques such as MRI in cancer [40] are promising and might be worth evaluating in inflammation as well.

As mentioned above, CXCR6 expression in RA was found prior to CXCL16 expression [38]. Until recently, CXCR6 expression was confined to lymphocytes. However, Ruth and colleagues recently claimed CXCR6 expression by synovial macrophages and migration of monocytes towards CXCL16 [41]. Obviously, this finding would have great consequences for our understanding of the role of CXCL16 and CXCR6, both in general and in RA. As monocytes would migrate towards CXCL16, this would imply that either a CXCR6 is expressed on monocytes, or another CXCL16 receptor exists. Moreover, it would imply that both receptor and ligand are present on the same cell. This by itself is not unique, as for instance CCL19 is secreted by mature DC that also express its receptor CCR7. Interestingly, the adhesive capacities of CXCL16 [42] suggest that expression of both a surface bound ligand and its receptor may play a role in macrophage to macrophage adhesion and subsequently in the maintenance of the synovial lining. However, neither on protein level (FACS), nor on mRNA level (PCR) could we detect CXCR6 expression (unpublished observations), while we consistently found high expression on SF lymphocytes and a stable expression on blood lymphocytes [33]. Since migration of myeloid cells towards CXCL16 has also not been confirmed by others, a role for CXCL16 in attracting myeloid cells in RA ST is still debatable. It is of the utmost importance to confirm or reject the hypothesis

that CXCL16 acts on myeloid cells. As also the role of CXCL16 in cell adhesion is not generally accepted one should be cautious in drawing conclusions regarding this topic. In conclusion, much is still to be further investigated on the role of CXCL16 and CXCR6 in RA joints.

RA is an independent risk factor for atherosclerosis and patients with RA have been shown to have an accelerated atherogenesis and [43-45], a condition that is driven by internalization of oxLDL, subsequent foam cell formation and local inflammation [46, 47]. Next to T cell attraction, CXCL16 has been proposed to act as a scavenger receptor for oxLDL [48]. As CXCL16 expression was found in atherosclerotic plaques 49, it was suggested to play a role in atherosclerosis. In addition, it was recently shown that hyperhomocysteinemia upregulates CXCL16 expression [50]. However, the exact role of CXCL16 in atherosclerosis is unclear. As membrane-bound CXCL16 contributes to internalization of oxLDL, it would seem likely that it enhances atherosclerosis though acting in favour of foam cell formation. It is therefore remarkable that decreased plasma CXCL16 levels were associated with coronary artery disease in humans [51]. In contrast, others found that elevated CXCL16 levels were correlated with coronary artery disease. In this study, CXCL16 was suggested to be a marker for atherosclerosis and acute coronary syndromes [52]. In addition, a polymorphism of CXCL16 was found to be associated with severity of coronary artery disease [53] , which unfortunately has not been confirmed in a second cohort yet. Surprisingly, targeted disruption of CXCL16 accelerated atherosclerosis in a murine model [54], suggesting that CXCL16 might have an atheroprotective role. The mechanism behind this effect however remained unclear, as mice lacking CXCL16 expression were less capable of internalizing oxLDL. This in turn would suggest that oxLDL uptake through CXCL16 does not act in favour of atherosclerosis, which rises against the common opinion that oxLDL uptake is a pro-atherogenic feature. The latter is supported by several previous studies [55-59]. The question that rises upon these results is whether oxLDL uptake by CXCL16 is functionally different from oxLDL uptake by other scavenger receptors. A recent study, showing that mice deficient of the scavenger receptors SR-A and CD36 were not protected against atherosclerosis but even had accelerated atherosclerosis [60], does not support this idea and even questions the role of scavenger receptors as a group. The pro's and contra's regarding a causal role for CXCL16 in atherosclerosis were recently discussed in an elegant review by Sheikine and Sirsjö [61]. When all currently available data on the role of CXCL16 are taken into account, it is highly debatable whether circulating CXCL16 levels can be related to atherosclerosis, which is in line with our data from chapter 7 [62, 63]. Although factors that act in favour of atherosclerosis have been described in RA [43, 64-66] and CXCL16 might play a role in its pathogenesis, it is unlikely that soluble CXCL16 plays a role in this susceptibility to atherosclerosis as soluble CXCL16 does not internalize oxLDL

and systemic CXCL16 levels are not elevated in RA [63], regardless of disease activity. Moreover, we did not observe any correlations between soluble CXCL16 levels and circulating levels of the well appreciated marker for cardiovascular disease apolipoprotein B (apoB) [67-69]. In general, soluble CXCL16 might still play a significant role in atherosclerosis through its ability to attract activated T cells, which may in turn be important for the maintenance of the local inflammatory response that is typical for atherosclerosis. Therefore more in depth research on the role of CXCL16 is warranted, to determine the role of both membrane-bound CXCL16 and soluble CXCL16. Nonetheless, the currently available data do not directly support neutralization of CXCL16 as a strategy to combat atherosclerosis, since it is still uncertain whether CXCL16 is a force to support or combat in atherosclerosis.

In summary, there is now substantial evidence supporting a role for CXCL16 and CXCR6 in the pathogenesis of RA. The exact contribution of this chemokine-receptor pair to inflammation in RA needs to be determined by further studies in the following years. Regarding the potential role of CXCL16 in atherosclerosis, things are currently becoming more complex rather than more clear, which makes any prediction on its potential as a therapeutic target almost impossible.

Concluding remarks

In the present thesis, we aimed to explore the potential of CCL18 and CXCL16 as a therapeutic target and as a clinical marker in RA. Considering the first goal, it is critical to understand the function of a molecule in health and disease. Regarding CCL18, this knowledge is far from sufficient and CCL18 is still more or less a black box. The currently available data on CCL18 do not directly lead to great optimism on its potential as a target in RA. Perhaps chances are better in SSc, especially as it might play a dual role in this disease. Although serum CCL18 did correlate with disease activity, it seems to be safe to conclude that CCL18 is not suitable as a single marker for RA disease activity. For CXCL16, there is no evidence supporting the use of soluble CXCL16 in serum as a clinical marker for follow-up purposes in RA. Whether CXCL16 is useful as a marker for atherosclerosis also remains to be elucidated. Meanwhile, it will be interesting to follow the discussion on CXCL16 as a atherogenic or atheroprotective agent in atherosclerosis. Although much more is to be learned about the function of CXCL16 and CXCR6, intervening in their interaction might be useful as a tool to combat RA. Whether this potential intervention would be best directed against CXCL16 or CXCR6 and which tools would be best suited for this purpose will be a future challenge for investigators in the field. Additional research is clearly needed to determine the potential of interfering with “traffic control” by means of targeting CXCL16, CCL18, or other chemokines in RA.

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chapter 9

SUMMARY

Rheumatoid arthritis (RA) is a chronic auto-immune disease which is characterized by joint inflammation, potentially leading to cartilage damage and bone erosions. As for many inflammatory diseases, a variety of leukocytes play a critical role in the pathogenesis of RA. T cells and antigen presenting cells (APC), such as dendritic cells (DC) and macrophages, are two groups of leukocytes which are believed to play a role in RA. These APC and T cells interact in order to initiate immune responses. This APC-T cell interaction is facilitated by T cell attracting chemokines that are secreted by APC. In this thesis, our aim was to investigate the potential role of two of relatively new T cell attracting chemokines, namely CCL18 and CXCL16, in the pathogenesis of RA.

In previous experiments, we started with a large panel of chemokines of which several were identified as potentially interesting in RA, including CCL18 and CXCL16. In *chapter 1*, we introduce CCL18 and CXCL16 as potential mediators in the pathogenesis of auto-immune diseases such as RA. In *chapter 2*, we show that *in vitro* monocyte derived DC (MoDC) from RA patients have a higher mRNA expression of the chemokines CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8 when compared with MoDC from healthy controls. Neutralization of TNF-α is now a successful therapeutic strategy in patients with RA and may have an effect on chemokine expression. Although the DC phenotype is not altered by TNF-α neutralization, MoDC matured in the presence of soluble TNF-α receptors secrete lower cytokine levels and have a lower mRNA expression of T cell attracting chemokines such as CCL18. In *chapter 3*, we show that CCL18 protein is strategically expressed in RA ST. As CCL18 might play a role in RA, we were interested in its regulation on protein level. In *chapter 3*, we demonstrate that CCL18 protein secretion is driven by IL-4 and IL-13, in strong synergy with IL-10. In contrast, stimulation with pro-inflammatory cytokines has no effect on CCL18 protein secretion *in vitro*. Interestingly, incubation with synovial fluid (SF) from RA patients significantly enhances the CCL18 inducing effects of IL-4, IL-13 and IL-10, suggesting another yet unknown CCL18 inducing factor is present in RA SF.

The chemokine receptor CXCR6, which is present on activated T cells, was recently shown to be abundant in RA SF. This prompted us to investigate the expression of its only ligand, CXCL16, in RA. In *chapter 4*, we demonstrate elevated CXCL16 expression in RA ST and SF of RA patients. Furthermore, we show that CXCL16 is functionally capable of attracting memory type T cells from RA SF, potentially contributing to T cell influxes in RA joints. In *chapter 5*, we further investigated the regulation of CXCL16 secretion in general and in RA. In this study, we show that monocyte differentiation by itself is the strongest inducer of CXCL16 expression and secretion *in vitro*. To a lesser extend, cytokines have a positive (IFN-γ) or negative (IL-4, IL-13, IL-10) effect on CXCL16 secretion. Although local CXCL16 expression is elevated in RA joints, regulation of CXCL16 in myeloid cells *in vitro* is not altered in RA patients.

As CCL18 and CXCL16 might play a role in the pathogenesis of RA, we were interested in their circulating levels and potential as clinical markers. In *chapter 6*, we show that serum CCL18 levels but not serum CXCL16 levels are elevated in RA patients. Moreover, we show that CCL18 levels significantly correlate with disease activity, while neither CCL18 nor CXCL16 levels correlate with joint damage. Neutralization of TNF-α *in vivo* results in decreased serum levels of both chemokines, independently of disease activity. Next to its chemokine function, CXCL16 also acts as a scavenger receptor for oxidized LDL (oxLDL), which may be of interest in RA, as patients with RA have an enhanced cardiovascular morbidity. In *chapter 7*, we show that serum CXCL16 levels do not correlate with other markers of atherosclerosis in patients with RA. In addition, we demonstrate that CXCL16 does not bind oxLDL in a liquid phase, suggesting that CXCL16 does not play a role in atherosclerosis through scavenging oxLDL in the circulation as was previously suggested. Together, our findings suggest that CXCL16 and CCL18 may be involved in the pathogenesis of RA, which is discussed in *chapter 8*. The extend of this potential role in RA and their potential as therapeutic targets remains to be elucidated by future studies, both in general and in RA.

Samenvatting

Reumatoïde artritis (RA) is een chronische auto-immuun ziekte die gekenmerkt wordt door ontsteking van de gewrichten, hetgeen kan leiden tot kraakbeen beschadiging en bot erosie. Zoals bij veel ontstekingziekten spelen verschillende leukocyten een belangrijke rol in de pathogenese van RA. T cellen and antigeen presenterende cellen (APC), zoals dendritische cellen (DC) en macrofagen, zijn twee soorten leukocyten waarvan gedacht wordt dat ze een rol spelen in RA. Deze APC en T cellen communiceren met elkaar en initiëren zo een immuun respons. Deze APC-T cel interactie wordt mogelijk gemaakt door chemokines die worden uitgescheiden door APC om T cellen aan te trekken. Het doel van dit proefschrift was de mogelijke rol van twee relatief nieuwe chemokines, CCL18 en CXCL16, in de pathogenese van RA te onderzoeken.

Tijdens eerdere experimenten startten we met een groot chemokine panel waarvan enkelen potentieel interessant bleken in RA, waaronder CCL18 and CXCL16. In *hoofdstuk 1* worden CCL18 en CXCL16 geïntroduceerd als potentiële mediatoren in de pathogenese van auto-immuun ziekten zoals RA. In *hoofdstuk 2* tonen we aan dat *in vitro* gekweekte DC ("monocyte derived DC" (MoDC)) van RA patiënten een hogere mRNA expressie hebben van de chemokines CCL17, CCL18, CCL19, CCL22, CCL3 and CXCL8 vergeleken met MoDC van gezonde controles. Het neutraliseren van TNF-α is een succesvolle behandeling van RA en verloopt mogelijkerwijs via effecten op chemokine expressie. Hoewel het DC fenotype niet wordt beïnvloed door remming van TNF-α, produceren deze MoDC wel minder cytokines en hebben zij een lagere chemokine expressie, o.a. van CCL18. In *hoofdstuk 3* laten we zien dat CCL18 op strategische plekken tot expressie komt in RA ST op eiwit niveau. Aangezien CCL18 een mogelijke rol speelt in RA waren wij geïnteresseerd in de regulatie van CCL18 op eiwit niveau. In *hoofdstuk 3* tonen we aan dat CCL18 eiwit secretie sterk afhankelijk is van IL-4 en IL-13, in synergie met IL-10. Stimulatie met pro-inflammatoire cytokines daarentegen heeft geen effect op CCL18 eiwit secretie *in vitro*. Incubatie met synoviaal vocht (SF) van RA patiënten leidt tot een significante toename van CCL18 secretie door IL-4, IL-13 en IL-10, hetgeen suggereert dat er nog een onbekende CCL18 stimulerende factor aanwezig is in RA SF.

De chemokine receptor CXCR6, welke tot expressie komt op geactiveerde T cellen, werd recent aangetoond op lymfocyten in RA SF, met hoge expressie. Hierop raakten wij geïnteresseerd in de expressie van zijn enige ligand, CXCL16, in RA. In *hoofdstuk 4* laten we zien dat CXCL16 expressie verhoogd is in RA ST en in SF van RA patiënten. Bovendien tonen we aan dat CXCL16 ook functioneel memory T cellen aantrekt uit het SF, hetgeen wellicht bedraagt aan T cel influxen in RA gewrichten. In *hoofdstuk 5* bestuderen we de regulatie van CXCL16 secretie gedetailleerder, zowel in het al-

gemeen als in RA. In deze studie laten we zien dat differentiatie van monocyten op zich de sterkste stimulus is voor CXCL16 expressie en secretie *in vitro*. In mindere mate hebben cytokines een positief (IFN-γ) of negatief (IL-4, IL-13, IL-10) effect op CXCL16 secretie. Hoewel locale CXCL16 expressie is verhoogd in RA gewrichten is de regulatie van CXCL16 in myeloide cellen *in vitro* niet veranderd bij RA patiënten.

Omdat CCL18 and CXCL16 een mogelijke rol spelen in de pathogenese van RA waren we geïnteresseerd in de spiegels van deze chemokines in de circulatie om zo tevens hun potentie als klinische markers te onderzoeken. In *hoofdstuk 6* tonen we aan dat serum CCL18 spiegels in tegenstelling tot CXCL16 spiegels niet verhoogd zijn bij RA patiënten. Bovendien laten we zien dat CCL18 spiegels significant gecorreleerd zijn met ziekte activiteit, terwijl noch CCL18 noch CXCL16 spiegels correleren met gewrichtsschade. Het neutraliseren van TNF-α *in vivo* resulteert in verlaagde serum spiegels van beide chemokines, onafhankelijk van ziekte activiteit. Naast zijn rol als chemokine heeft CXCL16 ook een functie als "scavenger receptor" voor geoxideerd LDL (oxLDL), hetgeen mogelijk interessant is in RA, aangezien patiënten met RA een verhoogde cardiovasculaire morbiditeit hebben. In *hoofdstuk 7* laten we zien dat serum CXCL16 spiegels niet correleren met andere markers voor atherosclerose bij patiënten met RA. Verder tonen we aan dat CXCL16 niet bindt aan oxLDL in oplossing, hetgeen suggereert dat CXCL16 geen rol speelt in atherosclerose middels het wegvangen van oxLDL uit de circulatie zoals eerder werd gesuggereerd in de literatuur. Samengevat suggereren onze bevindingen dat CXCL16 en CCL18 mogelijk een rol spelen in de pathogenese van RA, hetgeen wordt bediscussieerd in *hoofdstuk 8*. De omvang van deze mogelijke rol in RA en de potentie als therapeutisch target dient verder te worden bestudeerd in aanvullend onderzoek, zowel in het algemeen als in RA.

chapter 10

INTERNATIONAL PUBLICATIONS

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chapter 11

DANKWOORD

Mijn werkzaamheden zijn altijd op meerdere plekken geweest, waardoor je het voorrecht hebt met veel verschillende mensen te mogen samenwerken. Natuurlijk was dit proefschrift niet mogelijk geweest zonder jullie bijdrage, groot of klein. Het is bijna onmogelijk om iedereen hiervoor voldoende te bedanken, maar een aantal mensen waarmee ik samen heb mogen werken wil ik er graag uitlichten: Allereerst *Piet*; ik dank je voor het vertrouwen dat je als promotor in mij als onderzoeker hebt gehad. En natuurlijk ook de mogelijkheden die ik kreeg als beginnend arts op de polikliniek. Mede door jouw leiding is de afdeling reumatische ziekten een prettige plek om te werken en je te ontwikkelen. Ik kijk er dan ook naar uit om weer op het oude nest terug te keren voor het laatste deel van mijn opleiding. *Tim*, het is heel simpel: Zonder jouw enthousiasme destijds was ik er überhaupt nooit aan begonnen. Dank je voor de unieke kans om mee te mogen werken aan het vormgeven van een onderzoekslijn, die denk ik zeker erg succesvol zal worden de komende jaren. Ik denk dat onze toch verschillende manier van denken en werken uiteindelijk positief heeft uitgepakt gezien het resultaat. Overigens bedenk ik me hierbij wel dat dit geintje me een stage (lees: reis) naar Portugal heeft gekost, maar dat strepen we dan maar weg tegen de congressen. *Gosse*, bedankt dat ik mijn laatste jaar onder jouw supervisie op het TIL heb mogen doorbrengen. Ik heb in dat jaar ontzettend veel geleerd en ben erg blij dat je ook als mijn promotor hebt willen optreden. Ik weet zeker dat ik nog vaak ga terugdenken aan onze gesprekken wanneer ik in de kliniek weer in aanraking kom met (fundamenteel) onderzoek. Misschien dat ik nu ook eindelijk tijd heb om je huisje in Frankrijk eens te testen met een goede fles wijn.

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altijd het geduld op wist te brengen om mijn labwerk statistisch verantwoord te analyseren, voor zover wij statistiek daaromtrent überhaupt bijdragend acht(t)en. Veel onderzoekers op weinig vierkante meters leidt tot kruisbestuiving; zo kun je onze samenwerking denk ik het beste beschrijven, *Calin*. Ik vond het erg leuk met je samen te werken en hoop dit in de toekomst als reumatologen op klinisch of onderzoeks gebied nog zeker vaker te doen. Bedankt ook *Berry en Heidi* (lab interne geneeskunde) want zonder jullie hulp was het nooit gelukt om onze link tussen CXCL16 en oxLDL te leggen (danwel te ontkrachten).

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chapter 12

CURRICULUM VITAE

Twan van Lieshout werd geboren op 24 juli 1977 te Uden. In 1995 behaalde hij zijn atheneum diploma op het Macropedius College te Gemert (thans Comeniuscollege). Hij studeerde geneeskunde aan de Radboud Universiteit Nijmegen, waar hij in 2000 het doctoraal behaalde, gevolgd door het artsexamen in 2002. Tot 2006 verrichte hij onderzoek als promovendus op het laboratorium voor experimentele reumatologie naast werkzaamheden op de afdeling reumatische ziekten in het UMC St. Radboud te Nijmegen. Sinds 2006 is hij werkzaam als AIOS interne geneeskunde in het Jeroen Bosch ziekenhuis te 's Hertogenbosch (opleider dr. P.M. Netten) in het kader van de opleiding tot reumatoloog (opleiders prof. dr. P.L.C.M. van Riel en dr. M.J. Franssen).

COLOUR FIGURES

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Figure 5

CCL18 expression in normal and RA synovial tissue.

Panel A and B depict 2 sections of control synovium, where CCL18 expression is expressed in parts of the lining and some perivascular regions. Panel D and E depict 2 representative synovial sections from RA where CCL18 is present in the lining and perivascular regions. Panel C and F represent isotype controls on RA synovium and that from healthy individuals respectively.

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Figure 2

Enhanced expression of CXCL16 protein within RA synovia. CXCL16 was expressed in the thin synovial lining (arrowhead) of healthy synovia (A), but was much more pronounced by the hyperthrophic lining of RA synovia (B). Many cells present within the sub-lining of RA synovia express high levels of CXCL16 (B and C). Note that (cleaved) CXCL16 was also associated with filaments of the extracellular matrix (B). Staining of serial sections indicated that expression of CXCL16 (E) correlated with the presence of CD68+ synovial Macrophages (F). We also detected CXCL16 within some vessels (G). Staining serial sections for CD31 confirmed that these cells were endothelial cells (H). In addition, many lymphocyte aggregates contain CXCL16+ cells (I). Analysis of stained serial sections suggested that these CXCL16+ cells were CD68+ Macrophages (J) amidst CD45RO+ memory lymphocytes (K). Cryo-sections were stained for CXCL16 (A-C, E, G and I), CD68 (F and J), CD31 (H), or CD45RO (K), or matched control antibodies (D and I). All sections were counterstained with hematoxylin. Sections shown are representative for ST from 10 RA patients and 5 controls. (Original magnifications x 400 in A-F, and I-L; x 630 in G and H).

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Figure 4

Reduced CXCL16 expression upon successful anti-TNF treatment of RA patients. Before anti-TNF treatment, CXCL16 is strongly expressed within RA synovia (A and C). Expression of CXCL16 is severely reduced in clinically responding patients (B), but not in non-responding patients (D). Synovia were isolated before (A and C) and after (B and D) anti-TNF treatment. All cryo-sections were stained for CXCL16 and counterstained with hematoxylin. Control stainings were negative. Results are representative of 3 responding and 3 non-responding patients. (Original magnifications x 100).

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Figure 5

Enhanced expression of CXCL16 protease ADAM-10 by RA synovial macrophages. ADAM-10 negative peripheral blood monocytes were differentiated into Macrophages and analyzed by flow cytometry. A: CD14+ monocytes/ Macrophages were gated and stained with anti-ADAM-10 (bold lines) or isotype-matched control antibodies (thin lines). Dead cells were excluded by gating on propidium iodide-negative cells. Addition of RA SF to macrophages (day 3) increases their expression of ADAM-10 (B). After 2 days the percentage of ADAM-10+CD14+ macrophages was determined by flow cytometry. The mean and SD percentages from 2 donors are shown. Dead cells were excluded by gating on propidium iodide-negative cells. * = $p<0.05$ versus controls. ST from controls showed expression of both CXCL16 (C) and its protease ADAM-10 (D) in the thin synovial lining. In contrast, expression of both CXCL16 (F) and ADAM10 (G) was highly enhanced in the hypercellular synovial lining of RA ST. Frozen sections were stained for CXCL16 (C and F), ADAM-10 (D and G), or a matched control antibody (E), and counterstained with hematoxylin. Representative stainings are shown. See figure 1 for other definitions (Original magnifications x 400).

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Figure 5

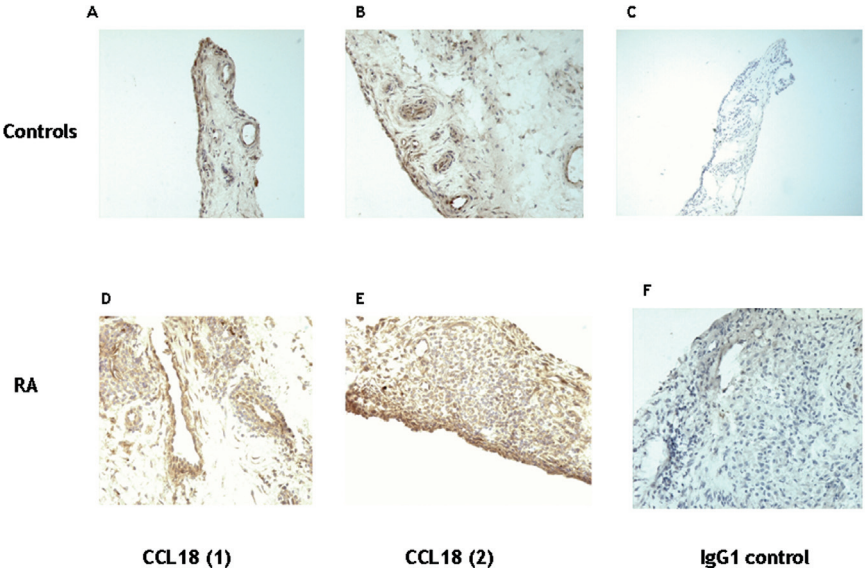

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Figure 2

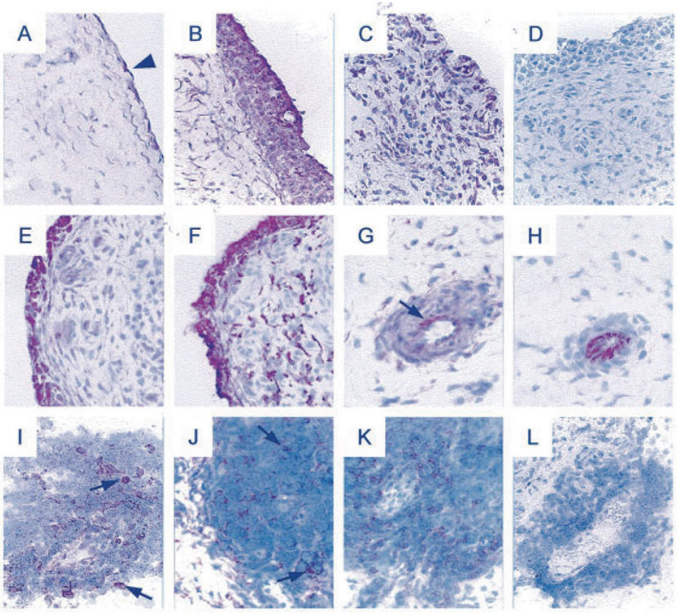

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Figure 4

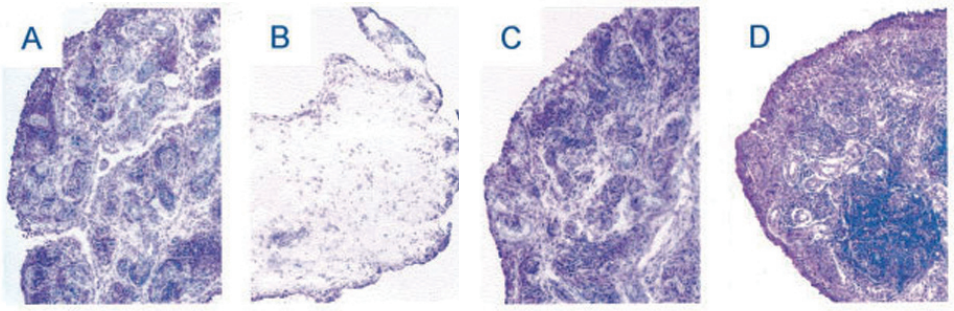

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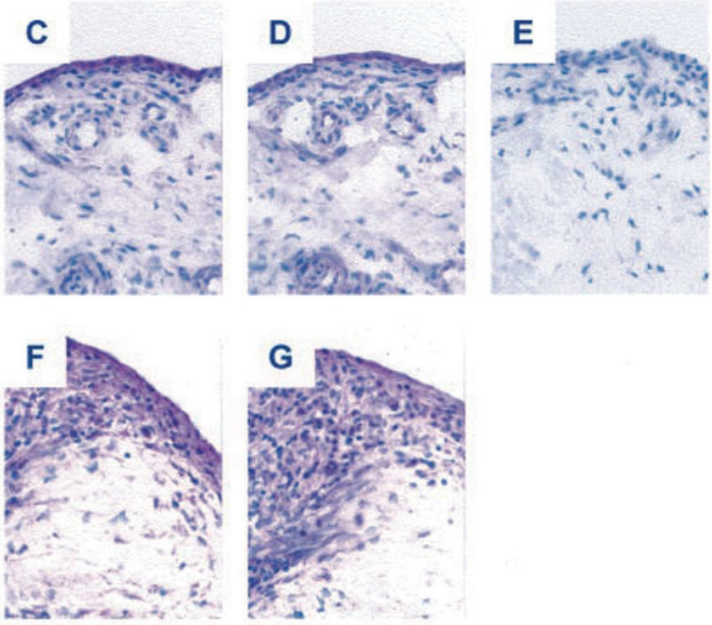
Figure 5



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